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PATHOGENICITY OF TYROSINASE-DEFICIENT MUTANTS OF STREPTOMYCES SCABIES[1]

(byline)

BY K. F. GREGORY AND E. B. VAISEY[2]

Abstract

Natural and X-ray induced mutations rendered strains of *Streptomyces scabies* simultaneously tyrosinase-deficient and unable to form a "brown-ring" in skim milk. These results and the presence of the tyrosinase reaction in each of 80 brown-ring positive streptomycetes tested were considered to indicate the identity of the brown-ring test and the tyrosinase reaction. All 12 tyrosinase-deficient mutants tested were virulent for Katahdin potatoes in controlled greenhouse tests.

Introduction

The characteristic of the potato scab producing organism, *Streptomyces scabies* (Thaxter) Waksman & Henrici, of forming a dark pigment when grown in ordinary nutrient media in contact with the air, was noted in Thaxter's original description of the species (10). Later workers found all pathogenic strains to be able to form a brown pigmentation on media containing proteins (6) or tyrosine (1). Taylor and Decker (9) observed a perfect correlation between the ability of a culture to produce typical lesions of potato scab and its ability to form a dark brown ring on separated milk. A similarly high (but not perfect) correlation was recently reported by Vaisey *et al.* (11) between the "brown-ring" test and pathogenicity of actinomycetes isolated from potato scab lesions. This relationship did not hold true, however, when the isolations were made from soil.

The dark brown pigment formed on protein media has been attributed to "tyrosine metabolism" (8). The ability to produce this pigment has been shown to be widely distributed among non-pathogens as well as pathogens (3, 8, 11, 12). Millard and Burr (7) reported that several of their pathogenic cultures failed to give a tyrosinase reaction but when a number of these

cultures were retested their pathogenicity could not be confirmed (1).   The possibility remains, therefore, that this property is a requisite for the pathogenicity of *S. scabies*.   This paper reports the results of experiments designed to test this point.

Materials and Methods

Isolation of Mutants

Streptomyces scabies, O.A.C. strains A26 and A30, originally isolated from potato scab lesions, were subjected to single spore isolations with the de Fonbrune micromanipulator.   The cultures were maintained on 1-oz. bottle slants of a synthetic asparagine–glucose agar medium[3].   Spore suspensions were prepared aseptically as needed by suspending the surface growth of a slant in 10 ml. of 1 : 5000 Tween "20"[4] solution and filtering it through a sterile No. 1 Whatman filter paper to remove mycelial fragments.

Spontaneous mutants were obtained by diluting spore suspensions to yield 40 to 100 colonies when 1 ml. of the dilution was spread over the surface of a medium prepared by mixing 10 ml. of sterile skim milk with 90 ml. of sterile, melted Difco nutrient agar.

Colonies which did not produce a brown pigment in this medium after five to six days' incubation were considered to be potential mutants and were inoculated directly into tubes of fluid skim milk.   If these failed to form a brown ring after 20 days' incubation they were transferred to slants of asparagine–glucose agar and tested for pathogenicity.   In addition, colonies producing a brown pigment on the milk agar were picked at random and inoculated into skim milk.

X-ray induced mutants were obtained by placing 5 ml. of a spore suspension in a small sterile Petri plate (4.5 cm. in diameter), the top of which was replaced by cellophane.   This was placed 8 cm. from the target of a General Electric DX-4.0 X-ray tube.   The X rays were produced at 85 Kv.P. and 5 ma.   The rays were unfiltered and had a H.V.L. of 0.7 mm. Al at 25 cm. f.s.d.   The intensity as measured in air was 1170 r. per minute.   One milliliter portions of the irradiated spore suspension were drawn off at intervals, diluted, and plated on the milk agar medium.   Mutant and non-mutant clones were isolated as described above.

All isolates were streaked onto Petri plates containing asparagine–glucose medium to test for sporulation and simultaneously onto the same medium + 0.04% L-tyrosine to test for darkening of this medium.

Pathogenicity Tests

Six brown-ring negative mutants derived from each of the two parent strains along with three brown-ring positive clones simultaneously isolated from each of the parents were tested for their ability to cause potato scab.

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[3]*Asparagine, 1.0 gm.; glucose, 5.0 gm.; KH_2PO_4, 0.5 gm.; $MgSO_4$, 0.2 gm.; $FeCl_3$, trace; distilled water, 1000 ml.; pH adjusted to 7.0.*
[4]*A wetting agent obtained from the Atlas Powder Company, Wilmington, Del., U.S.A.*

Tests were conducted in the greenhouse according to the methods developed by Jaques (5) as follows: Seven-inch pots were filled with a mixture of garden loam, peat, and sand in a 7:3:2 ratio. The pots were watered, covered with paper, and autoclaved at 15 lb. pressure for three hours. Scab-free seed pieces weighing not less than 35 gm. were cut from Katahdin potatoes. The pieces were allowed to suberize by exposure to air about 24 hr. They were then immersed in 1:1000 mercuric chloride for one-half hour and thoroughly rinsed with sterile distilled water. One surface-sterilized seed piece was planted per pot. Each test culture was incubated for seven days at 28°C. on asparagine–glucose 6-oz. bottle slants, the growth suspended in 1:5000 Tween 20 and diluted to 40 ml. One milliliter of each suspension was used to inoculate 100 ml. amounts of yeast–glucose[5] broth in 300 ml. Erlenmeyer flasks. These were incubated on a reciprocating shaker at 28°C. for 48 hr. The growth from one flask was aseptically mixed with the top three inches of soil in a pot. Non-inoculated controls were prepared in the same way using sterile broth.

All cultures were tested in triplicate and the pots randomized in the greenhouse. The pots were set in saucers to which water was added every two to five days as required. Tubers were harvested when the tops began to die (about three months).

Streptomycetes were reisolated from scab lesions from one tuber in each pot. Small portions of typical lesions, together with underlying non-infected tissue, were removed with a sterile scalpel and placed for two minutes in a test tube containing 1:1000 mercuric chloride. The lesions were then rinsed in sterile distilled water for five minutes, crushed in 1 ml. sterile water in a Petri plate, and streaked on the surface of milk agar plates. Following incubation at 28°C. colonies developed which could be rated according to their ability to cause browning of the medium. Eight to 10 colonies derived from each tuber were transferred to tubes of sterile skim milk to confirm the presence or absence of the brown-ring reaction.

Results

Spontaneously occurring mutants which lacked the ability to form a brown pigment when plated on milk agar, comprised about 0.2% of the total colonies. The age of the culture had no consistent effect on the proportion of these mutants. X-ray treatment appreciably increased the proportion of mutants with an exposure time of 30 min. yielding about 5% mutants. Ninety-four per cent of the suspected brown-ring negative colonies which were transferred to sterile skim milk failed to cause any browning of the milk. More than half of the mutant colonies isolated proved to be asporogenous on asparagine–glucose medium whereas none of the parental type brown-ring positive colonies lacked the ability to form conidia (Table I).

A perfect correlation existed between the brown-ring reactions of the mutant and parental-type clones and their ability to form dark brown discolorations

Yeast extract, 10 gm.; glucose, 5 gm.; M/20 KH₂PO₄–NaOH buffer at pH 6.8, 1000 ml.

TABLE I

CORRELATIONS OF "BROWN-RING" REACTION WITH
ASPOROGENESIS AND TYROSINASE ACTIVITY

Parent strain	"Brown-ring" reaction of colonies	Number of colonies isolated	Asporogenous colonies, %	Colonies causing darkening of tyrosine medium, %
A26	0	20	85	0
A26	+	10	0	100
A30	0	8	50	0
A30	+	9	0	100

of asparagine–glucose agar containing 0.04% L-tyrosine (Table I). All the brown-ring negative variants failed to form a brown pigment on this medium even on prolonged incubation.

Eighty diverse brown-ring positive streptomycetes isolated from soil and infected potato tubers (11) turned asparagine–tyrosine agar darker than the control medium lacking tyrosine. This pigment was considered to be melanin although no specific tests for melanin are available. After two or three weeks' incubation many of the cultures on the control medium produced a dark pigment similar in color to that produced on the asparagine–tyrosine agar. These pigments were indistinguishable with regard to solubility in pyridine, distilled water, absolute ethanol, acetone, and normal hydrochloric acid. Both pigments were also bleached by normal nitric acid and by potassium permanganate when followed by oxalic acid. These similarities suggested that the dark brown pigment on the control medium was also melanin, and it seemed possible that this melanin might have arisen through the action of tyrosinase on free tyrosine originating within the cell.

To test this hypothesis, several brown-ring positive streptomycetes were inoculated into fluid asparagine–glycerol medium and incubated on a reciprocating shaker for three days. These cells were harvested, washed, and ground in a Potter homogenizer. The protein was precipitated with 10% trichloracetic acid and separated by centrifugation. The clear solution was analyzed for amino acids by one dimensional filter paper chromatography (13). Spots corresponding in R_f value to cystine, asparagine, dihydroxyphenylalanine (dopa), and glutamic acid were identified.

The results of pathogenicity tests in the greenhouse are summarized in Table II. In no instance was scab encountered on tubers from non-inoculated control pots. Without exception, however, all mutants and parental type clones produced typical scab lesions (Fig. 1).

Reisolations of streptomycetes from scab lesions produced by brown-ring positive clones yielded only brown-ring positive cultures. Similarly, in most cases, only brown-ring negative cultures were reisolated from tubers inoculated with the mutant cultures. A notable exception was strain A26-k. Although

PLATE I
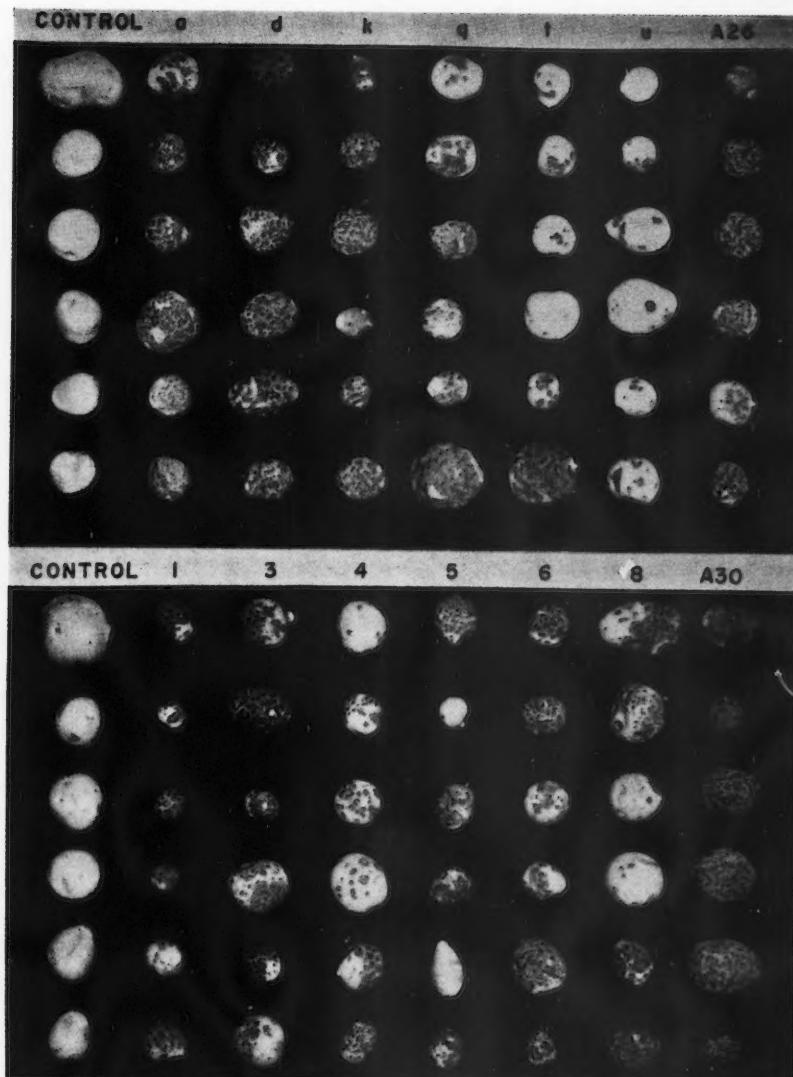
FIG. 1. Scab lesions formed on Katahdin potatoes inoculated with tyrosinase-producing strains of *S. scabies* A26 and A30 and six tyrosinase-deficient mutants derived from each.
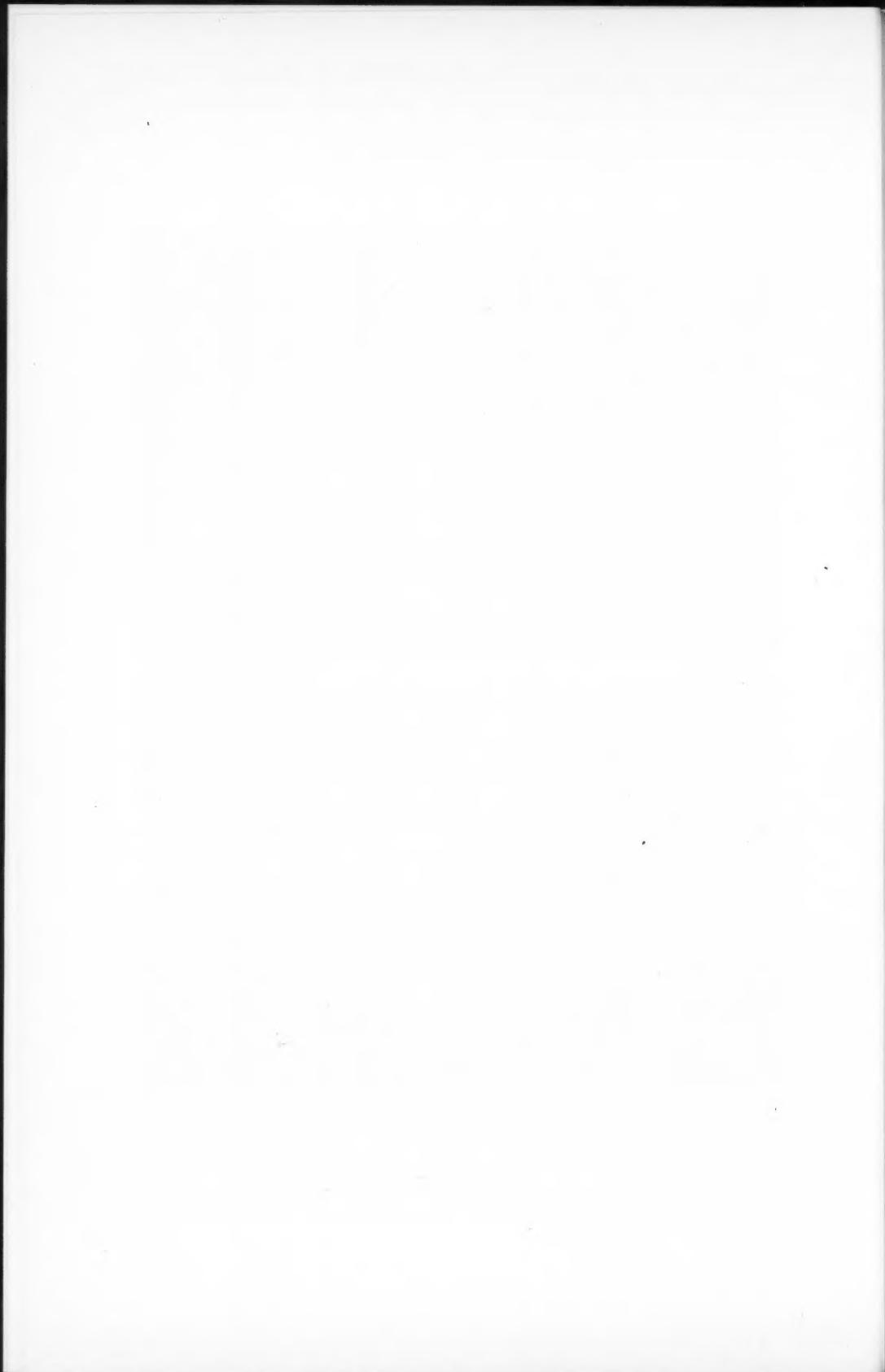
TABLE II

PATHOGENICITY OF BROWN-RING POSITIVE AND BROWN-RING NEGATIVE CLONES OF
Streptomyces scabies

Strain number	"Brown-ring" reaction	Sporogenesis	Per cent scab*	Per cent of reisolated streptomycetes brown-ring positive
No inoculum	—	—	0	—
Clones derived from strain A26				
A26-A	+	+	69	100
A26-C	+	+	68	100
A26-D	+	+	72	100
A26-a	0	+	64	0
A26-d	0	+	57	0
A26-k	0	0	83	100
A26-q	0	+	44	0
A26-t	0	0	18	0
A26-u	0	0	16	35
Clones derived from strain A30				
A30-III	+	+	54	100
A30-V	+	+	41	100
A30-VII	+	+	75	100
A30-1	0	+	52	37
A30-3	0	+	63	0
A30-4	0	0	40	15
A30-5	0	+	76	0
A30-6	0	0	42	0
A30-8	0	+	46	0

*L.S.D.; 5% point = 31.4, 1% point = 42.3.

the inoculum was an asporogenous brown-ring negative variant, isolations from all replicate pots yielded only sporogenous, brown-ring positive colonies. This culture also produced the highest incidence of scab of all the strains tested.

Discussion

The results indicate that the "brown-ring" reaction produced in skim milk by the strains of *S. scabies* tested was a manifestation of the tyrosinase reaction since single mutations rendered the progeny simultaneously brown-ring negative and tyrosinase-deficient. These results are considered to indicate the identity of the brown-ring formation to the tyrosinase reaction in the strains studied. The fact that every one of an additional 80 brown-ring positive streptomycetes turned the tyrosine medium darker than the control medium suggests that the brown-ring reaction in most, if not in all, cases indicates the presence of tyrosinase. The results do not rule out the possibility that some streptomycetes may produce a dark brown pigment other than melanin. The properties of the brown pigment produced by some cultures after prolonged incubation on tyrosine-free medium indicated, however, that it was the same as that produced on the tyrosine medium and

presumedly melanin.   A plausible explanation for the source of the pigment in these cases is that tyrosinase within the cells acted upon tyrosine originating from the degradation of cellular protein.   Since melanin is believed to arise by the oxidation of tyrosine via dihydroxyphenylalanine (dopa) (4), the evidence pointing to the presence of free dopa in the cells adds support to this explanation.   The tyrosinase-deficient mutants formed no brown pigment even upon prolonged incubation.

Preliminary respiration experiments with mutant strain A26-a indicated that this strain oxidized L-tyrosine but failed to oxidize L-dopa in contrast to brown-ring positive cultures which oxidized both (2).   The oxidation of tyrosine in this culture thus appeared to be by an alternate route to that followed as a result of tyrosinase action.   Tyrosinase is known to oxidize both tyrosine and dopa (4).

The reason for the high incidence of asporogenous cultures among the tyrosinase-deficient mutants is not apparent.   Since neither mutant nor parental-type colonies formed spores on the milk agar medium used for the initial isolation of clones there appears to have been no reason for an accidental bias in the random selection of brown-ring positive colonies from these plates.

The greenhouse tests clearly showed that all of the tyrosinase-deficient mutants were pathogenic.   Although some of these strains were as virulent as the tyrosinase-positive strains, some caused an incidence of scab which was significantly lower than that caused by the parental types.   These low virulent strains were also asporogenous, however, so the decreased virulence may not have been a result of the tyrosinase deficiency per se.   Indeed a calculation of X^2 from a contingency table based on the data in Table II revealed a significant correlation between sporogenesis and per cent scab produced by the strains tested.

The fact that most of the scab lesions from plants inoculated with brown-ring negative strains yielded pure cultures of brown-ring negative streptomycetes confirms that the lesions were caused by the tyrosinase-deficient cultures and not by tyrosinase-producing cells which may have arisen by back-mutation.   Repeated platings of mutant cultures failed to reveal a single instance of back-mutation to tyrosinase competence.   The reason for the aberrant behavior of mutant strain A26-k which produced scab lesions yielding only brown-ring positive colonies is not known.

The results reported here do not negate the value of the brown-ring test of Taylor and Decker (9) as an aid to selecting pathogenic from saprophytic streptomycetes in isolations from scabbed potato tubers.   They do show, however, that a positive brown-ring reaction per se (found to be identical to the tyrosinase reaction in the strains tested) is not related to virulence. Since the natural frequency of mutation to tyrosinase-deficiency is relatively high, it is considered likely that pathogenic brown-ring negative forms of S. scabies occur in nature.

Acknowledgments

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PRODUCTION OF POLYHYDRIC ALCOHOLS BY OSMOPHILIC YEASTS[1]

By J. F. T. Spencer and H. R. Sallans

Abstract

Cultures of osmophilic yeasts were isolated from fermenting honey, brood comb pollen, flower parts, and other sources and tested for glycerol production. Most of the strains tested produced one or more of the sugar alcohols glycerol, erythritol, D-arabitol, and mannitol, in quantities varying with the strain of yeast and the conditions of growth. Study of factors affecting the production of glycerol and D-arabitol by one strain showed that yields were influenced by the nitrogen source and by the rate of aeration. Increasing the concentration of yeast extract in the medium increased the rate of glucose utilization, the ratio of glycerol to D-arabitol, and the actual yield of glycerol. Addition of small quantities of urea, ammonium sulphate, ammonium tartrate, or ammonium phosphate gave similar results, and urea had the greatest effect. More vigorous aeration reduced the rate of glucose utilization, reduced the yield of ethanol, and increased the yield of glycerol, without affecting the D-arabitol yield. As much as 60% of the glucose dissimilated was converted to polyhydric alcohols.

Introduction

Yeasts and other microorganisms produce glycerol in small quantities during normal fermentation, and in the presence of sulphite or alkali there is an increase in the amount of glycerol produced by yeast. A fermentation process not requiring steering agents might be useful commercially, so there is still interest in organisms producing glycerol by a "normal" fermentation process.

The production of glycerol by organisms other than *Saccharomyces cerevisiae* was reported by Neish, Ledingham, and Blackwood (6) who patented a process for production of glycerol, 2,3-butanediol, lactic acid, ethanol, and formic acid by fermentation of glucose by *Bacillus licheniformis* (Ford's strain of *Bacillus subtilis*), and by McBee (4) who isolated a thermophilic, cellulose-fermenting bacterium which produced carbon dioxide, hydrogen, ethanol, formic acid, acetic acid, succinic acid, and glycerol. Nickerson and Carroll (7) found that *Zygosaccharomyces acidifaciens*, a yeast isolated from sour wine, and since reclassified as *Saccharomyces acidifaciens* by Lodder and Kreger-van Rij (3), produced a substance which they identified tentatively as glycerol, in yields of about 20% of the weight of sugar fermented. The production of other polyhydric alcohols by yeasts has not been reported before, though Binkley and Wolfrom (1) found traces of erythritol and D-arabitol in the fermentation residues from Cuban molasses, and suggested that these products were formed by the yeast.

The report by Nickerson and Carroll stimulated investigation of the fermentative properties of the osmophilic yeasts also classified in the *Zygosaccharomyces* spp. in the hope that an organism producing good yields of glycerol during normal fermentation might be found. However, the yields of glycerol actually recovered were considerably lower than were indicated by the analytical results, and it was found that part of the material reacting as glycerol was D-arabitol. A survey of osmophilic yeasts, collected from various sources, was made to find out what other products might be formed and some factors affecting the yields of glycerol and D-arabitol produced by one rapidly growing strain were studied.

Materials and Methods

Isolation of Cultures

Yeasts were isolated from fermenting honey, pollen stored in brood comb, from dried figs, flower parts, and a fermenting parsnip. Pieces of the source material were planted in tubes of broth containing 60% honey and 0.25% yeast extract. When growth was well advanced the cultures were streaked on plates containing the same medium plus 2.0% agar. Individual colonies were picked from these plates to slants of the same medium. These cultures were checked for purity by restreaking and by microscopic examination.

Testing of Cultures

All the isolates were tested for fermentative ability in a medium containing 20% glucose, 1% yeast extract, and 0.1–0.15% urea. Fifty milliliters of this medium were placed in a 500 ml. Erlenmeyer flask, sterilized, inoculated, and placed on a Gump rotary shaker operating at 230 r.p.m., with an eccentricity of one inch, at a temperature of 30° C. At intervals aseptic samples were removed and analyzed for reducing sugars and total polyhydric alcohols. Paper chromatograms were made from each sample to detect the different polyhydric alcohols and similar compounds. One rapidly growing culture producing mostly glycerol and D-arabitol was selected for use in a study of factors influencing yields of these two compounds.

Fermentation Conditions

To a basal medium containing 20% glucose, different nitrogen sources were added. All fermentations were conducted in 250 ml. or 500 ml. Erlenmeyer flasks, and shaken either on a New Brunswick shaker having an eccentricity of one-half inch, or on a Gump shaker with an eccentricity of one inch. Changes in amount of aeration were effected by varying the volume of medium in the flask. Samples of the broth were taken aseptically and analyzed for reducing sugars, total polyhydric alcohols, glycerol, D-arabitol, and ethanol.

Analytical Methods

All methods used were described previously by Neish (5).

Reducing sugars were determined colorimetrically with the alkaline copper reagent of Somogyi and arsenomolybdate reagent of Nelson.

Polyhydric alcohols were determined by oxidation with periodic acid and colorimetric determination of the formaldehyde formed with chromotropic acid reagent.

Glycerol and D-arabitol were separated on water–celite columns using a mixture of 25% benzene and 75% butanol, saturated with water, as a developing solvent, following the method of Neish (5).

Ethanol was determined by oxidation with acid potassium dichromate in a Conway microdiffusion cell and determination of the excess dichromate with potassium iodide and sodium thiosulphate.

Paper chromatograms were developed with a solvent composed of butanol, acetic acid, and water (5:1:2 by volume), according to Hough's (2) method, and reducing sugars, polyhydric alcohols, and similar compounds were detected with alkaline silver nitrate solution by the method of Trevelyan, Procter, and Harrison (9).

Results

Survey of Cultures

The products of three yeasts isolated from brood comb pollen are shown in Fig. 1. The compound having the low R_f value is probably a polysaccharide gum, known to be produced by these organisms. The same compounds were formed by named species of *Zygosaccharomyces*, as shown in Fig. 2. Traces of two compounds of high R_f value were produced as well. *Zygosaccharomyces acidifaciens* grew more slowly and formed smaller quantities of products. *Z. barkeri* and *Z. nussbaumeri* (not shown) produced the same compounds in the same yields as did *Z. richteri*.

In all, 79 strains of osmophilic yeasts, including those mentioned above, were tested for polyhydric alcohol production, and the results of this survey
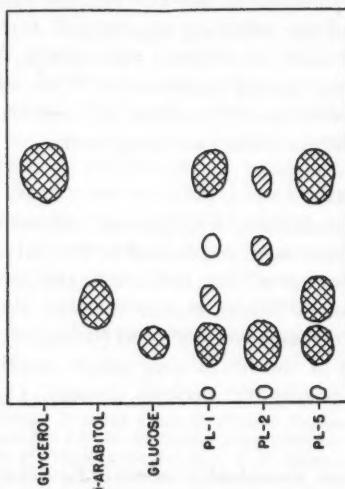
FIG. 1. Paper chromatogram showing fermentative products formed by three osmophilic yeasts isolated from brood comb pollen.
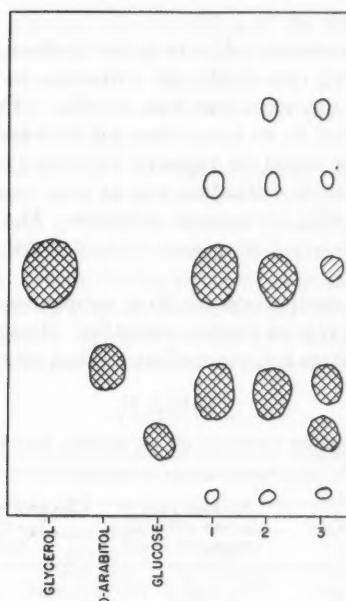
Fig. 2. Paper chromatogram showing fermentative products formed by named strains of *Zygosaccharomyces*. (1) *Z. mellis*, (2) *Z. richteri*, (3) *Z. acidifaciens*.

are shown in Table I.   About half of these produced mainly glycerol and D-arabitol.   This group included most of the fast growing cultures.   One exception was *Saccharomyces cerevisiae*, which utilized glucose much faster than the other strains and produced a small amount of glycerol but no D-arabitol.   All of the cultures producing mainly glycerol and erythritol were slow-growing strains.

TABLE I

CLASSIFICATION OF CULTURES ACCORDING TO PRODUCTS FORMED AND FERMENTATION TIME

Products formed*	Number of cultures fermenting a 20% glucose solution in:			Total number of cultures
	6 days or less	10–14 days	15 days and over	
Glycerol + D-arabitol	24	9	6	39
Glycerol + erythritol		4	9	13
Erythritol + D-arabitol		1	1	2
Erythritol			3	3
Glycerol	1	8	1	10
Glycerol + D-arabitol + erythritol	2	2	2	6
D-arabitol		5	1	6

*Mannitol was found during recovery of erythritol from fermentation liquor known to contain erythritol and glycerol.   Since it was not separated from residual glucose on the paper chromatograms the number of cultures which produce small amounts of mannitol is not known.

Effect of Nitrogen Source

The addition of ammonium sulphate to the medium, aerated at a low rate (Table II), increased the rate of glucose utilization to a maximum when the ammonium sulphate concentration was 0.05%.   The highest polyhydric alcohol yield was reached at an ammonium sulphate concentration of 0.025%.

Ammonium sulphate could be replaced by urea (Table III).   With urea the highest rate of glucose utilization was at urea concentrations equivalent in nitrogen to 0.10–0.20% ammonium sulphate.   The highest yield of polyhydric alcohols was reached at a urea concentration equivalent to 0.20% ammonium sulphate.

Table IV shows a comparison of three supplementary nitrogen sources. Urea gave the highest rate of glucose utilization, though ammonium sulphate and ammonium phosphate had some effect.   When no supplementary nitrogen

TABLE II

EFFECT OF AMMONIUM SULPHATE ON POLYHYDRIC ALCOHOL PRODUCTION*

$(NH_4)_2SO_4$, %	Average rate of glucose utilization, mgm./ml./day†	Glycerol + D-arabitol (as glycerol), mgm./ml.
0	7.6	63
0.025	8.8	65
0.05	10.2	41
0.10	8.8	36
0.20	9.4	29‡
0.40	8.3	23‡

*150 ml. medium containing 20% glucose and 0.4% yeast extract in 250 ml. Erlenmeyer flasks, on New Brunswick shaker at 160 r.p.m.   Temperature 30° C.
†Average of two flasks.   Rate of glucose utilization was determined after 17 days.   Polyhydric alcohols were determined at 29 days.
‡Polyhydric alcohols determined at 17 days as no further glucose utilization after this time.

TABLE III

EFFECT OF UREA ON POLYHYDRIC ALCOHOL PRODUCTION*

Urea, %	Equivalent $(NH_4)_2SO_4$, %	Average rate of glucose utilization, mgm./ml./day†	Final yield of polyols (as glycerol), mgm./ml.†
0	0	5.8	61
0.011	0.025	8.7	62
0.023	0.05	9.7	62
0.045	0.10	13.1	58
0.091	0.20	13.0	64
0.182	0.40	12.3	55

*150 ml. medium containing 20% glucose and 0.4% yeast extract in 250 ml. Erlenmeyer flasks, on New Brunswick shaker at 160 r.p.m.   Temperature 30° C.
†Average of two flasks.

source was used, little glycerol was formed and the yield of D-arabitol was high. Three times as much glycerol was formed when urea was added and the yield of D-arabitol was reduced. The increase in the yield of glycerol was much less with ammonium sulphate or ammonium phosphate, and the yield of D-arabitol was considerably reduced.

Further tests of supplementary nitrogen sources, with increased (1.0%) yeast extract concentration and more vigorous aeration showed similar effects on glucose utilization and polyhydric alcohol production (Table V). The highest rate of glucose utilization and next highest polyol yield were obtained in the presence of urea. With the higher level of yeast extract there was a large increase in the rate of glucose utilization by this yeast. Higher concentrations of supplementary nitrogen sources were used than in the previous experiments with some detrimental effects on the fermentations.

TABLE IV

EFFECT OF AMMONIUM PHOSPHATE, AMMONIUM SULPHATE, AND UREA ON GLYCEROL AND
D-ARABITOL PRODUCTION*

Supplementary nitrogen source	Average rate† of glucose utilization, mgm./ml./day	Final glycerol,† mgm./ml.	Final D-arabitol,† mgm./ml.	Ratio of glycerol: D-arabitol
None	7.8	6	94	0.064
Urea 0.114%	13.8	22	76	0.29
$(NH_4)_2SO_4$ 0.05%	10.8	14	56	0.25
$NH_4H_2PO_4$ 0.09%	11.5	7	66	0.11

*150 ml. medium containing 20% glucose and 0.4% yeast extract in 250 ml. Erlenmeyer flasks, on New Brunswick shaker at 160 r.p.m. Temperature 30° C.
†Average of two flasks.

TABLE V

EFFECT OF UREA, AMMONIUM SULPHATE, AMMONIUM PHOSPHATE, AND AMMONIUM TARTRATE
AS SUPPLEMENTARY NITROGEN SOURCES ON POLYHYDRIC ALCOHOL PRODUCTION*

Supplementary nitrogen source	Average rate of glucose utilization,† mgm./ml./day	Final polyhydric† alcohols (as glycerol), mgm./ml.
None	25	71
Urea	57	69
$(NH_4)_2SO_4$	37	46
$NH_4H_2PO_4$	18‡	30
$(NH_4)_2C_4H_4O_6$	57	63

*50 ml. medium containing 20% glucose, 1.0% yeast extract, and supplementary nitrogen sources equivalent to 0.25% $(NH_4)_2SO_4$ in 500 ml. Erlenmeyer flasks, on Gump shaker at 220 r.p.m.
†Average of two flasks.
‡Incomplete at eight days.

TABLE VI

EFFECT OF AERATION ON ETHANOL AND POLYHYDRIC ALCOHOL PRODUCTION*

Volume of medium in flask, ml.	Average rate of glucose utilization, mgm./ml./day	Final ethanol,† mgm./ml.	Final polyhydric alcohols (as glycerol),† mgm./ml.	Final glycerol, mgm./ml.	Final D-arabitol,† mgm./ml.
Experiment I					
200	62	51	35	—	—
100	61	40	43	—	—
50	56	19	56	—	—
25	53	1	78	—	—
Experiment II					
100	34	23	75	31	82
50	33	1	109	60	86
25	25	Nil	136	84	88

*Media: Experiment I, 20% glucose; 1% yeast extract; and 0.114% urea. Experiment II, 20% glucose; 1% yeast extract; and 0.20% urea. Fermentations run in 500 ml. Erlenmeyer flasks, on the Gump shaker at 230 r.p.m. Temperature 30° C.
†Average of two flasks.

Effect of Aeration

The yield of ethanol was greatly reduced and the yield of polyhydric alcohols was increased by increased aeration (Table VI). The increase was the result of an increase in the glycerol yield although the concentrations of all products reported for experiment II (Table V) are very high. This may be partially accounted for by evaporation losses, since each flask was sampled repeatedly and measurement of the volume changes was not attempted. Nevertheless, according to the analytical results for 50 ml. of medium approximately 60% of the glucose was converted to polyhydric alcohols, allowing for an estimated water loss of 20%.

Discussion

The osmophilic yeasts were found to produce high yields of polyhydric alcohols during normal fermentation. Glycerol, erythritol, D-arabitol, and mannitol are all produced by one or another of the strains tested in this laboratory. Pasteur discovered that small amounts of glycerol were produced by *Saccharomyces cerevisiae*, and Binkley and Wolfrom (1) suggested that yeast might produce traces of erythritol and D-arabitol, but this is the first report of the production of glycerol and other sugar alcohols in high yields by yeast during normal fermentation.

Many of the cultures tested could be placed in one of two groups, depending on the rate of glucose utilization and the polyhydric alcohols formed. One group formed mostly glycerol and D-arabitol and fermented glucose fairly rapidly. The other group, when grown under the same conditions, produced mostly erythritol, glycerol, and mannitol, and fermented glucose more slowly. However, it was found later that if the concentration of yeast extract is increased, the rate of glucose utilization by the latter group is more rapid.

Nitrogenous compounds have a marked effect on the behavior of the osmophilic yeasts. *S. cerevisiae* can be grown in media containing only ammonium chloride and urea as nitrogen sources (Prescott and Dunn (8)) while the osmophilic yeasts all require relatively high concentrations of yeast extract for good growth. Urea or ammonium nitrogen is also required when high yields of polyhydric alcohols are produced by these organisms. This indicates that the osmophilic yeasts require relatively large quantities of one or more growth factors, but the individual compounds required were not determined.

Both the osmophilic yeasts and *S. cerevisiae* produce glycerol and ethanol as major products of glucose metabolism, but in addition the osmophilic yeasts form six-, five-, and four-carbon polyhydric alcohols in high yields. The mechanism by which the sugar alcohols are formed is under study. Like *S. cerevisiae*, the osmophilic yeasts show a marked Pasteur effect, the amount of ethanol produced and the rate of glucose dissimilation decreasing with increasing aeration. Furthermore, there are large increases in the yield of glycerol. There was no significant change in the yield of D-arabitol with changes in aeration, which is some evidence that the mechanism of formation of these two compounds is considerably different.

The osmophilic yeasts, then, have several characteristics which have hitherto escaped recognition. They ferment concentrated glucose solutions rapidly, converting about 60% of the sugar to the polyhydric alcohols glycerol, D-arabitol, erythritol, and mannitol. Comparative yields of these alcohols may be altered greatly by selection of different strains and the precise control of growth conditions. Studies on the intermediary metabolism of these organisms may offer a new approach to our understanding of the alternate pathways of glucose dissimilation.

Acknowledgment

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THE EFFECT OF ANTIBIOTICS ON INFECTION OF WHEAT BY XANTHOMONAS TRANSLUCENS[1]

BY W. A. F. HAGBORG[2]

Abstract

Seedlings were grown in plexiglas culture vessels containing Shive's four-salt solution with trace elements added. Single antibiotics in various concentrations were added to the culture solution. Streptomycin and chloramphenicol increased the resistance of Thatcher wheat to infection by *Xanthomonas translucens* (S.J. & R.) Hagb., but additions of actidione (cycloheximide), griseofulvin, and neomycin failed to do so. The dry weight per plant was reduced by all of the antibiotics even when the antibiotic had no apparent effect on the rate or amount of disease development. The reduction in weight was least with neomycin and greatest with the higher concentrations of three forms of streptomycin tested. Chloramphenicol reduced dry weight appreciably even though infection was controlled. The induction of resistance appears to be closely related to phytotoxicity and to the sensitivity of the pathogen to the same antibiotic.

Introduction

Pramer (3) showed that the two actinomycete antibiotics, streptomycin and chloramphenicol, but not three others, chlortetracycline, oxytetracycline,[3] and neomycin, were readily taken up by the roots of cucumber seedlings and translocated to the leaves. That uptake and translocation occurred was indicated by the high antibiotic activity of the leaf tissues and of the sap exuded from the cut stems when the roots were in contact with a culture solution containing the antibiotic. The presence of streptomycin and chloramphenicol in the leaf tissues following absorption by the roots was established by paper chromatography.

Similarly Stokes (4) showed that griseofulvin can be taken up by the roots and translocated in wheat seedlings.

The present paper records the results of experiments in which wheat seedlings were allowed to take up streptomycin, chloramphenicol, and griseofulvin and were subsequently inoculated with a culture of *Xanthomonas translucens* (S.J. & R.) Hagb. Negative results with actidione (cycloheximide) and neomycin are also given.

Materials and Methods

Three experiments are reported here. In the first, actidione, chloramphenicol, and neomycin were tested; in the second, three forms of streptomycin, namely, dihydrostreptomycin, agristrep, and streptomycin sulphate; and in the third, griseofulvin.

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Contribution No. 1501 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.
[2]*Plant Pathologist, Plant Pathology Laboratory, Winnipeg, Manitoba.*
[3]*The trademark of Parke, Davis & Co., for the antibiotic chloramphenicol is Chloromycetin, of Lederle Laboratories Div., Am. Cyanamid Co., for the antibiotic chlortetracycline is Aureomycin, and of Chas. Pfizer & Co., for the antibiotic oxytetracycline is Terramycin.*

Freedom from contamination with metallic ions was ensured by the use of culture vessels constructed entirely of plastics. The plexiglas[4] culture vessels used were designed especially for these experiments and were constructed by Mr. R. H. Cunningham, Cereal Breeding Laboratory, Winnipeg. It is believed they will have a wide range of usefulness in plant growth studies. A vessel of this type is, for convenience, here named a "plasticon" (Fig. 1). It consists of an upper and a lower portion. The lower is a cylindrically shaped reservoir, a hollow cylinder, three inches in diameter, closed at the lower end. On its upper edge rests a disk which holds a central hollow cylinder, across the lower surface of which is stretched a netting of nylon marquisette cemented into the plexiglas by solution. All joins in the plexiglas are made by solution.

The dimensions of a plasticon are such that 10 to 15 cereal seedlings can be grown conveniently inside the inner cylinder. The roots grow through the netting into the culture solution and the plants can be lifted out intact on the upper portion during additions to, or replacement of, the culture solution. A cylinder of opaque paper is fitted over the reservoir of the plasticon to exclude light and so prevent the growth of algae. It can be removed readily for a visual examination of the roots. As an alternative, the plasticon may be painted on the outer surface.

In these experiments, Shive's four-salt solution was used. The pH value was 5.1 and the solution was supplemented with trace elements. It consisted of distilled water, 4 Imp. gal.; KH_2PO_4, 4.72 gm.; $Ca(NO_3)_2.4H_2O$, 16.08 gm.; $MgSO_4.7H_2O$, 8.56 gm.; $(NH_4)_2SO_4$ (anh.), 1.44 gm.; HBO_3, 0.016 gm.; $MnSO_4$, 0.016 gm.; and $ZnSO_4$, 0.016 gm. Freshly dissolved ferrous sulphate was added to the plasticons to provide an iron concentration of 3 μgm./ml. The griseofulvin was dissolved by refluxing for one hour. The remaining antibiotics dissolved readily to the required concentrations.

Work followed the procedure of Brian (1) in that the seedlings were allowed to develop a small root system before the antibiotic was added to the culture solution. To achieve uniformity in procedure, the seed was placed, on "zero day", between moist filter papers in a Petri dish for 24 hr. at a temperature of 20°C., then placed on the netting in the plasticons containing as much culture solution as was necessary to cover the nylon netting. The seed was allowed to sprout for 72 hr., after which the culture solution was replaced with one containing the antibiotic at the required concentration (Fig. 2).

Inoculation was effected by means of numerous pin pricks, throughout the length of the leaf, with a multiple point inoculating needle[5] dipped in the fresh growth of Xanthomonas translucens (culture 3074) originally isolated from a collection of bacterial black chaff on the glumes of wheat at Swan River, Manitoba. The inoculation was performed seven (Expt. 1), eight (Expt. 2), or 10 (Expt. 3) days after sowing and thus three, four, or six days

[4]Methyl methacrylate synthetic plastic.
[5]Consisting of a sheaf of several brass pins mounted on a handle.

after first exposure to the antibiotic.   Incubation was at a mean temperature of 20°C. (range 9–32°C.).   The symptoms of infection were observed 10 days after inoculation and growth was terminated for oven-drying on the 17th day (Expt. 1) or on the 22nd day (Expt. 2).

At the termination of the test the tops of the plasticons were removed and the roots rinsed in distilled water.   The plants were allowed to air-dry for three days, after which the roots were severed from the tops at the level of the nylon netting and the remains of the old testa and endosperm were removed and discarded.   Both the tops and the roots were then oven-dried at 98°C. for 24 hr., cooled, and weighed.

Effect of Antibiotics on Infection

Addition of streptomycin or chloramphenicol to the culture solution increased the resistance of Thatcher wheat seedlings to infection by *X. translucens*, but additions of actidione, griseofulvin, or neomycin had little or no effect.

In the inoculated controls, water-soaking was apparent around the margin of the wounds within four days of inoculation time.   A moderate infection . developed by the fifth day and by the 10th to 14th day the water-soaked area had become yellowish and translucent.   When actidione and neomycin were used the progress of the disease was similar to that in the controls but when streptomycin was used much less infection developed and when chloramphenicol was used none was visible, the wounds having the same appearance as those of the wounded, but uninoculated, controls (Table I).

Phytotoxicity

Wherever an antibiotic was added to the culture solution, even when the antibiotic had no apparent effect on disease development, the dry weight per plant was less than that of the controls (Table I).   The reduction in weight was least with neomycin and greatest with the higher concentration of the three forms of streptomycin.   Chloramphenicol also greatly reduced the dry weight.

Different phytotoxic effects occurred with different antibiotics.   Strepto-mycin caused distinct blanching that varied in distribution and intensity with different forms and concentrations of the antibiotic.   That the blanching was caused by a scarcity of chloroplasts was established by centrifugation (700 r.p.m.) of portions of blanched and control secondary leaves ground lightly in an isotonic sucrose (0.8 M) solution.   When approximately equal portions of the two suspensions were examined microscopically only two leucoplasts and no chloroplasts were found in the streptomycin-blanched leaves as compared with 504 chloroplasts in the controls.   Chloramphenicol caused a chlorosis that was more uniform in distribution and less pronounced in degree than that caused by streptomycin.

FIG. 1.   Two plasticons, the one assembled and the other open to illustrate the details of construction.

FIG. 2.   A series of plasticons in use.
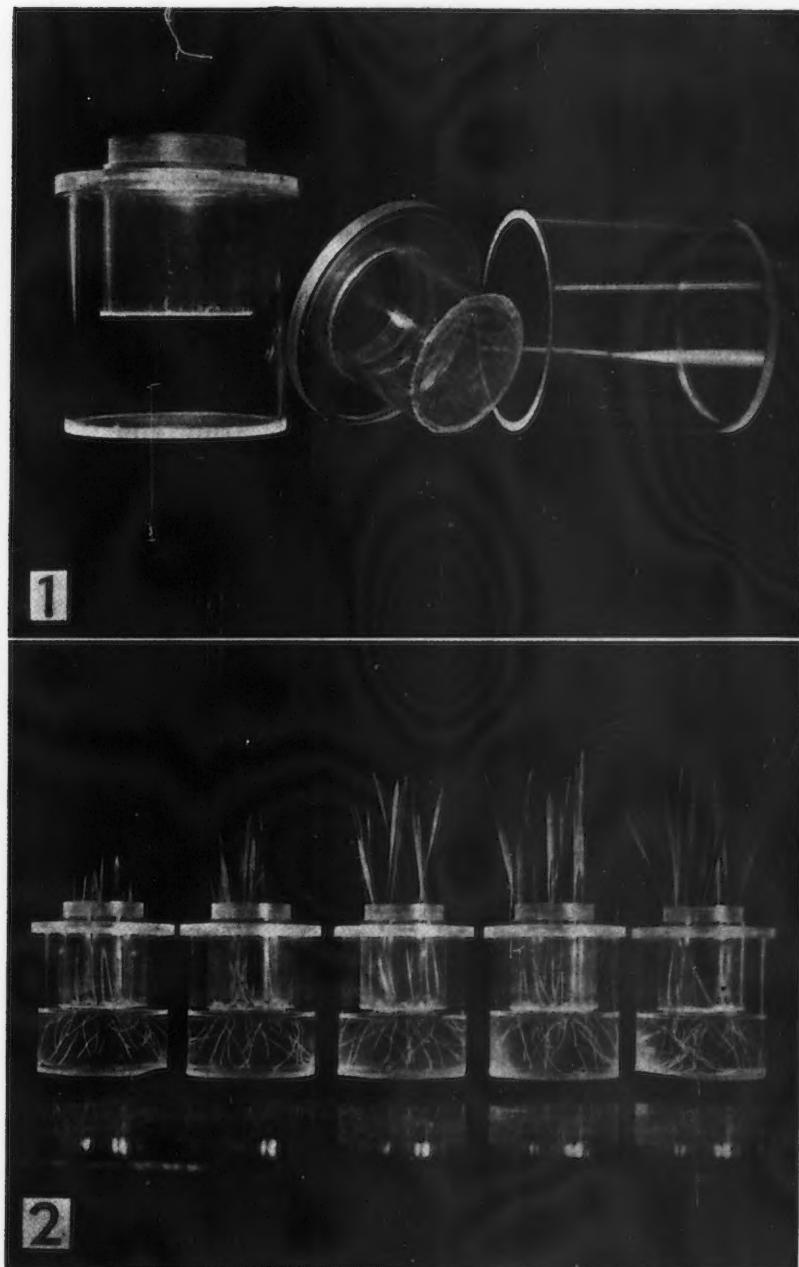
TABLE I

EFFECT OF ANTIBIOTICS ON INFECTION BY *X. translucens* AND ON DRY WEIGHT

Antibiotic	Concentration, μgm./ml.	Proportion of seedlings infected	Dry wt., gm.*				Dry wt., % of control
			Roots	Tops	Total	Diff. from control	
Expt. 1							
Actidione	4	9/9	0.0080	0.0400	0.0480	0.0410	53.9
	2	10/10	0.0150	0.0520	0.0670	0.0220	75.3
Chloramphenicol	50	0/10	0.0040	0.0220	0.0260	0.0630	29.2
	25	0/10	0.0060	0.0280	0.0340	0.0550	38.2
Neomycin sulphate	100	10/10	0.0100	0.0610	0.0710	0.0180	79.8
	50	10/10	0.0170	0.0610	0.0780	0.0110	87.6
Control	0	10/10	0.0210	0.0660	0.0890	0.0000	100.0
Pricked but uninoculated	0	0/10	0.0260	0.0710	0.0970	−0.0080	109.0
Expt. 2							
Dihydrostreptomycin (sulphate)	100	0/23	0.0014	0.0160	0.0174	0.1271	12.0
	50	2/24	0.0167	0.0558	0.0725	0.0720	50.2
Agristrep	100	11/22	0.0028	0.0205	0.0233	0.1212	15.4
	50	6/22	0.0118	0.0443	0.0561	0.0884	38.8
Streptomycin sulphate	100	8/24	0.0125	0.0317	0.0442	0.1003	30.6
	50	22/23	0.0335	0.0900	0.1235	0.0210	85.5
Control	0	22/22	0.0395	0.1050	0.1445	0.0000	100.0
Pricked but uninoculated	0	0/21	0.0417	0.1093	0.1510	−0.0065	104.5
Expt. 3							
Griseofulvin	ca. 20	20/20	Severe retardation of root system				
Control	0	20/20	Satisfactory growth and development				

In Experiment 1, the dry weights given are for single determinations and in Experiment 2 for the mean of two determinations.

Root dwarfing was evident with both actidione and griseofulvin. Griseofulvin caused a clubbing of the roots such as that described by Stokes (4). A second effect was a tendency towards chlorophyll production in the seminal roots in a band between the central area containing xylem elements and the outer cortical cells. That the green tissue contained chloroplasts was established microscopically in mounts made from a centrifuged suspension in 0.8 M sucrose of a ground root. Green chloroplasts were moderately abundant. An extract of the green roots studied in a Hilger spectrophotometer had absorption bands similar to those of chlorophyll extracted from a green leaf. Although the greenness of the roots was not observed when the other antibiotics were used nor in the control plants it appears probable that the chlorophyll production was in part, at least, attributable to a stimulation from light absorption through the surface of the culture solution. A similar production of chloroplasts was described and illustrated by Percival (2) in the aboveground adventitious roots of wheat.

Additional Results

In a fourth experiment, griseofulvin at a concentration of 6 μgm./ml. caused less retardation of growth than at 20 μgm./ml., and showed no evidence of inhibition of infection. Further confirmation that griseofulvin had no

inhibitory effect on infection by *X. translucens* was obtained in a fifth experiment set up as a demonstration for the Annual Meeting of the Canadian Society of Microbiologists at Winnipeg in June, 1955. In that experiment, griseofulvin was ineffective at 20, 10, 5, and 1 μgm./ml. Streptomycin and chloramphenicol again inhibited infection at 25 and at 50 μgm./ml. although, for some unknown reason, streptomycin did not do so' at 100 μgm./ml.

In a sixth experiment, chloramphenicol was used in a duplicate series of concentrations at 50, 25, 20, 10, 5, 2, and 0 μgm./ml. In it inhibition was complete at 50 and 25 μgm./ml. At 20, a trace of infection was present on one plant out of 12 and the infection became proportionately greater in the series until it reached 100% at 2 μgm./ml.

Discussion

It is evident that resistance to *X. translucens* can be induced by the absorption of streptomycin and chloramphenicol through the root system. The term "resistance" as used in plant pathology appears to have ample breadth of meaning to embrace the infection-inhibiting condition found in a plant following the uptake of an antibiotic. Nevertheless, because to specialists in various fields of pathology the word may have special connotations, it should be stated here that a heritable condition is not implied.

TABLE II

WIDTH OF ZONE OF INHIBITION IN NUTRIENT AGAR PLATES SEEDED WITH *Xanthomonas translucens* SURROUNDING PAPER DISKS 6.5 MM. IN DIAMETER AND CONTAINING ANTIBIOTICS* (17 ISOLATES IN DUPLICATE)

Antibiotic	Approximate quantity per disk, μgm.	Mean width of zone
Actidione	30	0
	10	0
	1	0
Chloramphenicol	10	12.9
	1	3.6
Neomycin	10	3.2
	1	1.6
Streptomycin	10	9.6
	1	6.4
Griseofulvin	0.27	0
	0.10	0
	0.01	0

*To ascertain that approximately the required weight of antibiotic was present in each disk, a standardized procedure was adopted for moistening and drying the disks. The moisture-holding capacity per disk was first determined and the concentration of each antibiotic adjusted according to its assay value to leave the required residue of dried antibiotic in the disk. The disks were held in the antibiotic solution for 15 sec., drained on glass, freed of drops of solution, then placed to dry in warm Petri dishes. They were held in a desiccator overnight and stored in a closed vial at 12° C.

Neither is the term "induced resistance" as used here necessarily equivalent to the "acquired resistance" of medical pathology. The two antibiotics obviously affect the physiology of the plants. This is indicated by the chlorosis they produce, but there are no grounds for assuming that they result in the production of antibodies.

The inhibition of bacterial infection may be primarily due to the antibiotic content of the plant tissues. When the five antibiotics were tested against 17 cultures of *X. translucens* in paper disk sensitivity plates, wide zones of inhibition surrounded the streptomycin and chloramphenicol, but not the actidione or griseofulvin. Narrow zones surrounded the neomycin disks (Table II). It is thus quite possible that the inhibition by streptomycin and chloramphenicol was a direct action by the antibiotic on the pathogen. This type of action is further suggested by the finding of Pramer (3) who, as stated above, identified both streptomycin and chloramphenicol by paper chromatography in the leaf tissues of cucumber plants that had been allowed to absorb these antibiotics through their roots. It appears likely that the phenomenon of resistance to *X. translucens* induced in the wheat plant by these two antibiotics is directly attributable to their presence in unchanged form, dissolved in the cell sap.

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THE EFFECT OF A NEW ANTISEPTIC
(1,6-DI-4'-CHLOROPHENYLDIGUANIDOHEXANE)
ON SKIN FLORA[1]

BY G. E. MYERS,[2] W. C. MACKENZIE,[3] AND K. A. WARD[4]

Abstract

A tincture and an aqueous solution containing 0.5% of 1,6-di-4'-chlorophenyl-diguanidohexane were tested *in vivo* as preoperative skin antiseptics. Skin biopsies were taken from various operative sites before and after a measured time of exposure to the antiseptic. Various periods of exposure were employed. The antiseptic was neutralized immediately at the end of the exposure time and bactericidal activity was tested by a standardized series of culture procedures. Tested under these conditions both the 0.5% aqueous solution and the 0.5% tincture possess marked skin disinfecting properties against a wide range of microorganisms.

Introduction

During an investigation of the biological properties of a series of poly-guanides Davis *et al.* (1) found that one compound, a bisguanide, having the formula 1,6-di-4'-chlorophenyldiguanidohexane, had outstanding antibacterial properties. Preliminary investigations by these workers, both *in vitro* and in laboratory animals, demonstrated that the antibacterial activity is exerted against a wide range of vegetative bacteria, both Gram-positive and Gram-negative. The compound is only slightly active against bacterial spores. The activity is not inhibited by body fluids but is antagonized by egg yolk. Toxicity is very low when the compound is given orally to laboratory animals. No interference with phagocytosis by human leucocytes was noted. Although the compound has no true systemic antibacterial activity it was found highly active in mice in artificial wounds infected with hemolytic streptococci. Two salts of the bisguanide are available, these are the diacetate and the dihydrochloride. The diacetate being of greater solubility is the more convenient for laboratory testing.

The present paper deals with the first *in vivo* tests of this agent as a preoperative skin antiseptic for humans. For the sake of brevity the manufacturers'* code name AY5312 will be used to represent the compound throughout this report. The diacetate salt of the compound was used throughout this investigation.

Materials and Methods

The method for testing the skin disinfecting ability of AY5312 is a modification of that employed by Walter (2) for testing alkyl-dimethyl-benzyl

ammonium chloride.   Walter's technique involves swabbing the operative area three times with the antiseptic "alternating with three changes of sponges saturated with 70 per cent alcohol."   A skin biopsy is then taken from the site, placed in Ringer's solution, and subsequently cultured in dextrose broth. Absence of growth is attributed to the antibacterial activity of the agent being tested.   Using this technique Walter reported that 73% of the biopsies showed no growth after treatment of the skin with alkyl-dimethyl-benzyl ammonium chloride.

Certain weaknesses are apparent in this technique.   First, failure of growth from any biopsy is attributed solely to the action of alkyl-dimethyl-benzyl ammonium chloride regardless of the fact that alternate swabbing of the skin surface with 70% alcohol was employed.   Second, failure of growth for biopsies taken after treatment of the skin with antiseptic is attributed solely to the antiseptic, although no control is included to determine whether growth would have taken place in the absence of the antiseptic.   Third, no mention is made of the time during which the antiseptic was in contact with the skin organisms.   Fourth, simple dilution is the only attempt made to stop the action of the disinfectant when the biopsy is placed in culture medium.

In view of these weaknesses we have modified the technique as follows. Preoperative preparation of the skin consists of preliminary swabbing with commercial ether for the purpose of simple cleansing.   Following this the first skin biopsy is taken and immediately placed in a small screw-capped vial containing a special neutralizing medium.   Culture of this tissue fragment will serve as a control to determine the bacterial flora of the skin prior to antiseptic treatment.   The operative site is next thoroughly swabbed with AY5312 (no other antibacterial agent included at this step in the procedure). After a measured interval of time a second skin biopsy is taken from a site adjacent to the first.   This tissue fragment is immediately placed in a second vial containing sterile neutralizing broth.   The vials labelled A and B respectively are immediately sent to the laboratory where they are placed in a 37° C. incubator.

Specimens are incubated 24 hr., then examined microscopically and subcultured to blood agar medium.   Blood agar plates are incubated 24 hr., then examined.   Visible growth is studied macroscopically and microscopically and all organisms identified.   If no growth develops on the blood agar medium by the end of 24 hr. incubation, the original broth culture is again plated to blood agar and incubated for 24 hr.   If no growth develops by the end of this incubation period, the original broth culture is inoculated to sterile nutrient broth and this medium is incubated for 24 hr.   If there is still no evidence of growth the organisms are considered dead.

The possibility exists that antiseptic may be transferred to the culture medium by way of the tissue fragment and thus prevent growth in vial B. To investigate this possibility each vial which failed to show growth after the routine incubation previously described was inoculated with the organisms

which grew in vial A for the same specimen. In no case did such organisms fail to grow profusely when inoculated into vial B, thus proving that the possible carry-over of antiseptic is insufficient to prevent growth.

Neutralizing Broth Medium

For the purpose of accurately measuring the bactericidal activity of any skin disinfectant during a standard period of exposure it is necessary to employ some means to stop the disinfecting action at the end of the exposure period. In the case of AY5312 Davis (1) demonstrated that egg yolk antagonizes the antibacterial action of the agent. Brain heart infusion broth containing egg yolk serves the purpose of neutralizing the disinfectant and at the same time provides an excellent medium for the growth of microorganisms.

The medium is prepared as follows. The yolks of six eggs are aseptically separated from the whites and added to 600 ml. of sterile Difco brain heart infusion broth. The mixture is emulsified and 20 gm. of kaolin is stirred in. The medium is allowed to sit overnight at refrigerator temperature (7° C.) and then clarified by filtering through ordinary qualitative filter paper. The filtrate is then sterilized by filtration through a Seitz E-K pad, and dispensed in 4 ml. volumes in sterile screw-capped vials.

Our preliminary experiments with this medium demonstrated that not only does it support the growth of the common skin organisms on initial isolation but in general such growth is more profuse than in brain heart infusion broth without egg yolk.

Experimental

Bacterial Flora of Cleansed Skin

During the course of this investigation 302 biopsies were studied. Half of these, 151, represent tissues taken after simple cleansing of the skin surface with ether and before antiseptic treatment. It is of. interest to note the bacterial flora of the skin from various regions of the human body under these conditions. Table I gives the results of the study of 148 biopsies of which the anatomical region of origin is known. Of special significance is the fact that of 148 specimens taken, a total of 16 failed to grow when incubated, i.e., over 10% of the biopsies taken gave no growth when no disinfectant was used. In no instance did a biopsy taken after disinfectant treatment give rise to growth when the biopsy taken before treatment was negative. Thus, if biopsies had been taken after disinfectant treatment only, this 10% failure of growth would have been attributed to disinfectant activity.

Aqueous Solution of AY5312 (0.5%)

An aqueous solution containing 0.5% AY5312 was tested using the procedure previously described. A total of 69 specimens (two biopsies each) was studied. Of these, one specimen was contaminated with an aerobic spore-bearing bacillus, and six specimens showed no growth in the biopsy taken prior to disinfectant treatment. Thus, 62 specimens were suitable for the

TABLE I

BACTERIAL FLORA OF SKIN BIOPSIES FOLLOWING PREPARATION WITH ETHER

	Source of specimen						
	Abdomen	Inguinal region	Leg and thigh	Back	Arm	Chest	Axilla
	No. of specimens investigated						
	85	21	26	3	4	8	1
	Number of isolations						
Micrococcus pyogenes var. *aureus* (hem.)*	20	6	4	0	0	3	1
Micrococcus pyogenes var. *aureus* (indiff.)†	26	8	7	1	2	1	0
Micrococcus pyogenes var. *albus*	37	6	2	0	1	2	0
Diphtheroid bacillus	13	4	3	0	1	1	0
Streptococcus viridans	5	1	1	0	0	1	0
Escherichia coli	1	0	0	0	0	0	0
Aerobic spore-bearing bacillus	4	1	4	0	0	1	0
No growth	3	4	7	2	0	0	0

* *(hem.)* =hemolytic.
† *(indiff.)* =non-hemolytic.

TABLE II

SKIN DISINFECTING ACTIVITY OF AY5312 (0.5% AQUEOUS SOLUTION) BASED ON TIME OF EXPOSURE

Exposure time, sec.	Number of specimens showing growth	
	Before exposure	After exposure
30	4	2
60	5	2
120	5	4
180	25	6 (24%)
240	23	1 (4.3%)

evaluation of the disinfecting ability of AY5312. The results of these studies are given in Table II. Of 25 specimens tested when the exposure time was 180 sec., six only (24%) showed viable organisms after exposure. Of 23 specimens showing growth prior to exposure for 240 sec., one only (4.3%) showed viable organisms after exposure. It is important to note that in no instance in this investigation did the biopsy taken after exposure give rise to growth of organisms other than those isolated prior to treatment.

Table III gives a more detailed picture of the results presented in Table II. Here the bacterial flora is shown together with the number of times each organism was isolated from specimens taken before disinfectant treatment for specified periods of exposure and the number of strains of each kind of organism

TABLE III

SKIN DISINFECTING ACTIVITY OF AY5312 (0.5% AQUEOUS SOLUTION) BASED ON TIME OF EXPOSURE AND ORGANISMS ISOLATED FROM BIOPSIES

	Exposure time, sec.									
	30		60		120		180		240	
	Isolations before	Viable after	Isolations before	Viable after	Isolations before	Viable after	Isolations before	Viable after	Isolations before	Viable after
Micrococcus pyogenes var. *aureus* (hem.)	2	1	2	1	0	0	9	5	4	0
Micrococcus pyogenes var. *aureus* (indiff.)	0	0	0	0	2	1	11	1	13	1
Micrococcus pyogenes var. *albus*	2	1	3	1	3	3	3	0	5	0
Diphtheroid bacillus	0	0	0	0	1	1	3	0	4	0
Streptococcus viridans	0	0	0	0	0	0	2	0	2	0

which remained viable despite disinfectant treatment. Thus *Micrococcus pyogenes* var. *aureus* (hem.) was isolated nine times from specimens taken prior to disinfectant exposure when the exposure period was 180 sec. Of the nine strains isolated five remained viable despite exposure to the disinfectant.

Tincture of AY5312 (0.5%)

Tincture of AY5312 containing 0.5% of the active ingredient was tested. A total of 82 specimens (164 biopsies) was studied. Of these, four were contaminated and 10 showed no growth prior to disinfectant treatment. Thus 68 specimens were suitable for evaluation of the disinfectant. The results of these tests are given in Table IV. When the exposure time was 180 sec., 25 specimens taken prior to disinfectant treatment showed growth, of these four only (16%) remained viable after disinfectant treatment. When the exposure time was 240 sec., 24 specimens taken prior to disinfectant treatment showed growth, of these two only (8.3%) remained viable after treatment.

Table V gives the detailed picture of the results presented in Table IV. *Micrococcus pyogenes* var. *aureus* (hem.) was isolated 10 times from specimens taken before disinfectant exposure of 240 sec. Of these 10 strains, two only survived disinfectant treatment.

During the course of this investigation no evidence of any skin irritation caused by AY5312 was observed in the 151 subjects studied.

TABLE IV

SKIN DISINFECTING ACTIVITY OF AY5312 (0.5% TINCTURE)
BASED ON TIME OF EXPOSURE

Exposure time, sec.	Number of specimens showing growth	
	Before exposure	After exposure
120	19	6 (32%)
180	25	4 (16%)
240	24	2 (8.3%)

TABLE V

SKIN DISINFECTING ACTIVITY OF AY5312 (0.5% TINCTURE) BASED ON EXPOSURE TIME
AND ORGANISMS ISOLATED FROM BIOPSIES

Exposure time, sec.	120		180		240	
	Isolations before	Viable after	Isolations before	Viable after	Isolations before	Viable after
Micrococcus pyogenes var. *aureus* (hem.)	4	2	5	1	10	2
Micrococcus pyogenes var. *aureus* (indiff.)	12	2	8	2	3	0
Micrococcus pyogenes var. *albus*	4	0	17	1	11	0
Diphtheroid bacillus	1	1	5	1	8	0
Streptococcus viridans	1	0	0	0	3	1
Escherichia coli	0	0	1	0	0	0

Discussion

From the results obtained in these experiments it is obvious that AY5312 in 0.5% concentration in aqueous solution or tincture has decided skin disinfecting activity. The range of antibacterial activity includes *Micrococcus pyogenes* var. *aureus* (hem. and indiff.), *Micrococcus pyogenes* var. *albus*, Diphtheroid bacillus, *Streptococcus viridans*, and *Escherichia coli*. The skin disinfecting ability of AY5312 is enhanced when the exposure time is lengthened. Exposure to the aqueous solution for three minutes results in no growth in 76% of the specimens tested, while exposure for four minutes results in no growth in 95%. Exposure to the tincture for three minutes results in no growth in 84% of the specimens taken, while exposure for four minutes results in absence of growth in 91% of the specimens.

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COMPARISON OF METHODS FOR ASSESSING THE ANTIGENIC RESPONSE TO PERTUSSIS VACCINE[1]

By E. M. Taylor, P. J. Moloney, and D. B. W. Reid

Abstract

Five methods which might be used for measuring the effectiveness of pertussis vaccines were compared using three vaccines. The methods did not yield the same relative results. It is highly probable that a mechanism other than humoral antibodies plays an important part in the mouse protection test since by this test the vaccines were graded differently than by a test depending on passive protection.

Introduction

In the course of work on pertussis vaccine a comparative study was carried out of a number of methods which might be used for measuring antigenic response. At present pertussis vaccines which are distributed for human use must fulfill certain antigenic requirements as measured by the official National Institutes of Health mouse protection test (6). It has been assumed that the test is a measure of the constituent of the vaccine which confers protection against whooping cough, though there is no worthwhile evidence to support this assumption. A test depending on passive protection to an intracerebral challenge of living *Hemophilus pertussis* might be expected to give similar results to the mouse protection test. Such a test has not been used heretofore for grading pertussis vaccines. Recently Evans (1) has described a method for determining the relative potency of pertussis vaccines in terms of agglutinin-producing power. In this study three vaccines were compared using the above three methods, together with two other methods which might possibly be used for the same purpose. The first of these was a test for the presence of histamine-sensitizing factor described by Parfentjev and Goodline (8) and more recently investigated by Parfentjev (7) and by Maitland, Kohn, and Macdonald (4). The latter authors discussed the possibility of its use for assessing the immunizing potency of pertussis vaccine. The other method was a test described by Malkiel and Hargis (5) for a factor which markedly enhances sensitization to serum proteins and other proteins, as manifested by anaphylactic shock.

This study was of necessity concerned with the relationships which exist among these five methods, not with their possible use in grading the anti-whooping cough effectiveness of pertussis vaccines.

Vaccines Used

The vaccines which were used as test material were fluid culture suspensions. One of the vaccines No. 801 was derived from four strains of *H. pertussis*. It was inactivated and detoxified by means of formaldehyde. The two other

vaccines were derived from a single strain of *H. pertussis* (strain 18943) which was different from any of the four strains used in the preparation of No. 801. Part of the fluid culture from which these two vaccines were derived was inactivated and detoxified by means of formaldehyde as in the case of No. 801. This vaccine is referred to as "unconcentrated vaccine (strain 18943)". To prepare the third vaccine a further portion of the fluid culture was adjusted to pH 4.0 with HCl and let stand at room temperature for two weeks. After this period of standing, sedimentation was so complete that a clear supernatant to the amount of 95% of the original volume could be siphoned off. The precipitated microorganisms were washed three times with sterile distilled water. At this stage the suspension was nontoxic by the standard toxicity test. A concentrated suspension in physiological saline (500 billion/ml.), adjusted to pH 8.3, was heated at 56° C. for 30 min. since it was found that heating under these conditions produced a marked reduction in pyrogenic activity. This vaccine is referred to as "heated vaccine (strain 18943)".

Pyrogen tests were carried out on the three vaccines. Pyrogenic activity of pertussis vaccine is a property which may or may not influence antigenicity; it most probably is a factor in reactions which follow inoculation of the vaccine. Table I shows the maximum average rise in temperature of groups of three rabbits following intravenous injection of a 15 billion dose of each vaccine.

TABLE I

PYROGEN TEST OF PERTUSSIS VACCINES

	Average maximum rise in temperature
Vaccine No. 801	2.9° F.
Unconcentrated vaccine (strain 18943)	2.8° F.
Heated vaccine (strain 18943)	1.7° F.

Methods and Results

N.I.H. Mouse Protection Test

In this test a single intraperitoneal injection of vaccine is given to mice and two weeks later the inoculated mice are challenged intracerebrally with living *H. pertussis*. Results are summarized in Table II. These indicate that unconcentrated vaccine (strain 18943) had significantly higher protective activity than either vaccine No. 801 or "heated vaccine"; and that there was no marked difference in the protective activity of these latter two.

To determine whether the protection induced by the vaccines was of a transient nature, a second challenge dose was given two weeks after the first to all survivors. It is obvious from the results in the table that the majority of mice which had received vaccine withstood a second challenge dose.

TABLE II

COMPARISON OF RESULTS OF MOUSE PROTECTION TESTS, TWO WEEKS AFTER INTRACEREBRAL CHALLENGE*

	Dose of vaccine (billion per ml.)	Vaccine injected 14 days before first challenge			Vaccine injected 3 days before challenge	
		No. mice	% survivors after 1st challenge	% survivors after 2nd challenge†	No. mice	% survivors
Vaccine No. 801	2.5	14	79	90	17	41
	0.62	13	54	72	—	—
	0.16	15	7	—	—	—
Unconcentrated vaccine (strain 18943)	2.5	17	94	100	17	71
	0.62	15	87	100	—	—
	0.16	16	31	100	—	—
Heated vaccine (strain 18943)	2.5	15	80	100	16	81
	0.62	15	40	67	—	—
	0.16	15	27	75	—	—

*Challenge dose (10^6 organisms) killed 11 out of 11 untreated mice.
†Second challenge dose (10^6 organisms) given to the survivors of the first challenge 15 days after first challenge.

Evans and Perkins (2) have shown that a single intraperitoneal dose induced protection to an intracerebral challenge dose of virulent *H. pertussis* in 70% of mice three days after the injection of vaccine, at a time when circulating specific antibodies were not detected. This observation was confirmed by challenging groups of mice three days after injection of vaccine. Results are shown in Table II.

Passive Protection Test

Serum for the passive protection test was prepared by injecting each vaccine into a group of 30 mice. Each mouse received two doses of 2.5 billion organisms, with an interval of two weeks between injections. Ten days after the second dose all the mice were bled out and sera of each group were pooled. The pooled sera were tested by injecting normal mice intraperitoneally with serum three hours before intracerebral challenge. The amounts of serum used and the number of mice surviving 14 days after challenge are shown in Table III. The results show that the serum from mice which had received vaccine No. 801 conferred greater protection than the other two sera.

TABLE III

PASSIVE PROTECTION OF UNVACCINATED MICE AGAINST INTRACEREBRAL
CHALLENGE* WITH VIRULENT *H. pertussis*

Vaccines used for preparation of sera	Mice surviving with the following dose of serum†			
	0.4 ml.	0.2 ml.	0.1 ml.	0.05 ml.
Vaccine No. 801	9/10	5/10	0/10	0/10
Unconcentrated vaccine (strain 18943)	2/10	3/10	2/10	0/10
Heated vaccine (strain 18943)	2/10	1/10	0/10	1/10

*Challenge dose (10^5 organisms) killed 10 out of 10 untreated mice.
†A dose of 0.8 ml. of serum from unvaccinated mice failed to protect any of 10 mice.

Test of Agglutinin Production

Following the procedure of Evans and Perkins (1) groups of female white mice were given two intraperitoneal injections of each of the vaccines at three concentrations with an interval of two weeks between injections. On the 10th or 11th day following the second injection the mice were anaesthetized and bled out. A pooling was made of 0.1 ml. of sera from mice which had received the same dosage of vaccine. The pooled sera were tested for agglutination with live pertussis culture using both Evans culture AGI at the recommended density of 30 billion and also the culture used for intracerebral challenge at a density of 10 billion. Mixtures of serum and organisms were incubated at 37° C. for one hour and the test read after the mixtures had stood overnight at room temperature. The results which are summarized in Table IV show that the serum from mice which had received vaccine No. 801 gave appreciable

TABLE IV

AGGLUTININ TITERS* OF SERA FROM GROUPS OF MICE INOCULATED WITH TWO DOSES OF PERTUSSIS VACCINE

	Evans culture AGI 30 billion per ml.			Challenge culture 18323 10 billion per ml.		
	10 billion vaccine	2.5 billion vaccine	0.62 billion vaccine	10 billion vaccine	2.5 billion vaccine	0.62 billion vaccine
Vaccine No. 801	800–1600	400–800	< 50	1600–3200	400–800	<50
Unconcentrated vaccine (strain 18943)	<100	<100	<100	<100	<100	<50
Heated vaccine (strain 18943)	<100	< 50	< 50	200–400	50–100	<50

*Reciprocals of serum dilutions.

agglutination with both cultures. Serum from mice which had received heated vaccine gave low-titer agglutination with the challenge culture, whereas serum from mice which received unconcentrated vaccine possessed no detectable agglutinins.

Test for Histamine Sensitizing Factor

The procedure used in this test was that described by Parfentjev (8). Female mice weighing 21 gm. were injected intraperitoneally (16 billion per dose) with two of the vaccines, and challenged five days later by intraperitoneal injection of graded doses of histamine diphosphate. Results summarized in Table V indicate that there was no appreciable difference between the two vaccines.

TABLE V

SENSITIZATION OF MICE TO HISTAMINE BY PERTUSSIS VACCINE

Dose of histamine diphosphate, mgm./kgm.	Mice dead in 18 hr. after injection of histamine		
	Control (not immunized)	Vaccine No. 801	Heated vaccine (strain 18943)
1600	2/8		
800	0/8		
400	0/8		
200	3/10		
100		7/8	8/8
50		7/8	8/8
20		4/8	5/8
10		0/7	0/9

TABLE VI

ENHANCEMENT OF SENSITIZATION OF MICE TO HORSE SERUM BY PERTUSSIS VACCINE

	Dose: 8.75 billion vaccine + 0.03 ml. horse serum			Dose: 8.75 billion vaccine	
	No. mice injected	Mice dead in 18 hr. after i.v. dose 0.1 ml. horse serum		No. mice injected	No. mice dead 18 hr. after i.v. dose 0.1 ml. horse serum
		No. mice	% of total		
Vaccine No. 801	18	1	6	9	0
Unconcentrated vaccine (strain 18943)	18	17	94	6	0
Heated vaccine (strain 18943)	18	9	50	6	0

0.03 ml. horse serum injected intraperitoneally into each of six mice. After 14 days one of six mice died in 18 hr. following intravenous injection with 0.1 ml. horse serum.

Test for the Factor which Enhances Sensitization to Horse Serum

In this test the procedure of Malkiel and Hargis (5) was followed, namely female white mice (13.5 to 15.5 gm.) were each injected intraperitoneally with a mixture of 8.75 billion pertussis vaccine and 0.03 ml. of horse serum.   Two weeks later the mice were challenged intravenously with 0.1 ml. of horse serum. The results summarized in Table VI indicate that though vaccine No. 801 produced no apparent effect, the other vaccines exercised an enhancing effect on sensitization with horse serum.   Results included in the table show that horse serum was nontoxic for the mice.

Discussion

The results of this study have been summarized in Table VII, where the effectiveness of the vaccines prepared from strain 18943 are expressed in terms of vaccine No. 801, as judged by each of the five methods investigated.   It is clear from the table that there are differences in the effectiveness of the vaccines but that these differences may vary according to the method of testing.   The mouse protection test, for example, shows that the "unconcentrated vaccine" is definitely superior in strength to vaccine No. 801 and that the "heated vaccine" is similar in strength to No. 801, whereas the agglutination and passive protection test indicate that both the "unconcentrated" and the "heated" vaccines are inferior to No. 801.   Further, as judged by sensitization to horse serum, vaccine No. 801 has no effect, whereas both of the other vaccines enhance sensitivity markedly.   From the data presented possible differences in results of agglutination and passive protection tests cannot be judged.

These observations are consistent with the assumption that there are at least three different constituents of pertussis vaccine which are revealed by the methods studied.

The mouse protection test and the passive protection test are applications respectively of active and passive immunization.   In the former a single

TABLE VII

COMPARISON OF METHODS

Test	Ratio of effectiveness of unconcentrated vaccine to vaccine No. 801	Ratio of effectiveness of heated vaccine to vaccine No. 801	Effect No. 801
Mouse protection	3.4*	1.2	Protection
Passive protection	0.5	0.3*	Protection
Agglutination	<0.25*	<0.25*	High titer agglutination
Histamine sensitization	—	1.3	Sensitization
Horse serum sensitization	>1*	>1*	No enhancement

*Significantly different from 1.

intraperitoneal injection of vaccine is given to mice and later the animals are challenged by intracerebral injection; in the latter test, serum from mice which had received two injections of vaccine is injected into normal mice and these are challenged intracerebrally.   Hence it might have been assumed that the mechanisms of protection were the same for both tests.   However, the results suggest that this is not the case, but rather that there is a different mechanism of protection operating in each method.   Since in the case of the passive protection test, protection can be presumed to depend upon antibodies, it is likely that a mechanism other than that of humoral antibodies plays an important part in the mouse protection test.   This conclusion is in agreement with results reported by Evans and Perkins (2)*.

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*Evans and Perkins (3) have stated in the summary of a paper which appeared after the preparation of the present communication that "It has been shown that two distinct immune response effects are produced in mice after a single intraperitoneal injection of pertussis vaccine.   The first is of an interference type; it is transient, it occurs rapidly, reaches its maximum effect in about 10 days and then quickly fades.   The second is of an antibody type; it occurs slowly reaches a high level in about 20 to 40 days when it shows no evidence of waning."

TAXONOMY OF LOPHOMONAS N. GEN.[1]

By Thomas P. Galarneault and Einar Leifson

Abstract

A study has been made of 25 strains of Gram-negative rod-shaped bacteria with lophotrichous flagella and no effect on carbohydrates. These bacteria are often found in human stools and blood, and in water of various kinds. Except for the flagellation, they are very similar to *Alcaligenes* species. The flagella are very characteristic, resembling those of spirilla. The wave length of the polar flagella averages 3.10 μ with an average amplitude of 1.08 μ. One strain, H-261, is genetically unstable and mutates through various intermediary stages to a genetically stable peritrichous type. The peritrichous (or nonpolar) flagella have several curves and a wave length of about 1 μ. The group showed considerable somatic and flagellar antigenic heterogeneity. All the strains studied appear sufficiently similar to be classified into one species. The name *Lophomonas alcaligenes* n. gen. is suggested for the group.

Introduction

Among the Gram-negative heterotrophic rod-shaped bacteria which do not attack carbohydrates are three distinct morphological types with peritrichous, polar lophotrichous, and polar monotrichous flagella. This fact has been known for over half a century but a satisfactory classification of the organisms is still wanting. The genus *Alcaligenes* is generally recognized as having peritrichous flagella and the physiologically related bacteria with polar flagella should not be included in this genus. Unfortunately, many bacteriologists completely disregard flagellation and label any Gram-negative heterotrophic rod which does not attack carbohydrates as *Alcaligenes*. Further confusion results from the use of conventional liquid fermentation media for determination of carbohydrate metabolism. Oxidative metabolism of carbohydrates may go undetected when liquid peptone media are used, and strains of *Pseudomonas*, *Achromobacter*, etc. not infrequently receive the label *Alcaligenes*.

The purpose of this paper is to describe a fairly homogeneous group of bacteria with lophotrichous flagella which at present appears to be taxonomically homeless. Many cultures of these organisms are found in stock culture collections with the label *Alcaligenes* sp., *Alcaligenes fecalis*, and occasionally, *Vibrio alcaligenes*.

Materials and Methods

The 25 cultures studied were obtained from a variety of sources over a period of several years and are listed in Table I.

The method of Hugh and Leifson (5) was used to determine carbohydrate metabolism. All carbohydrates and alcohols were sterilized by Seitz filtration and added aseptically to the sterile melted semisolid agar. Duplicate tubes were inoculated and one of these overlaid with a $\frac{1}{4}$ in. layer of sterile petro-

latum. Incubation was routinely at 30° C. Conventional methods were used with the other physiological reactions studied.

Bacto-Penassay base agar was used to test antibiotic sensitivity. Heavily seeded pour plates were prepared and five disks (Desi-Disks, National Bio-test Inc., and Bacto-Sensitivity Disks) impregnated with the various antibiotics were placed on each plate and incubated at 30° C. for 24 hr. A Fisher–Lilly zone reader was used to determine the degree of growth inhibition.

The antigens for immunization and serological study were prepared in the conventional manner by growing the cultures on agar slopes, suspending the growth in saline, and diluting the suspension to about 1 billion cells per ml. For H agglutination formalin was added to the antigen suspension to a final concentration of 0.5%. For O agglutination the tubes of antigen were suspended in boiling water for two hours. Antisera were prepared in rabbits by inoculating the live antigen in increasing doses at two day intervals for six injections. One week after the last injection the titer was checked. Further injections were made if the H titer was less than 1 : 5120. The animals were bled from the heart, the blood allowed to clot, and the serum stored at −20° F. H agglutination was determined after two hours at 52° C., and O agglutination after four hours at 52° C., followed by incubation at 37° C. overnight.

Several attempts to discover lysogenic strains were unsuccessful.

TABLE I

SOURCES OF CULTURES

Culture No.	Source	Other data
H-17	Human stool	Fulton No. 539. Isolated with *Salmonella*
H-31	Human stool	Fulton No. 2445. Diarrheal stool of child
H-32	Human stool	Fulton No. 3046
H-37	Water	Fulton No. 2686. Well water.
H-124	Human stool	Fulton No. 2120
H-131	Human stool	Goldin, Mt. Sinai Hospital, Chicago. No. 4444
H-133	Human stool	Goldin, Mt. Sinai Hospital, Chicago. No. 4579
H-185	Human stool	Friewer, Ill. Pub. Health. No. 10091SSR1
H-212	Human blood	Friewer, Ill. Pub. Health. No. 10958BC1
H-226		Nat. Col. Type Cult. No. 1347
H-260	Hay	Variant of ATCC No. 8461 with polar flagella
H-261	Hay	Variant of ATCC No. 8461 with peritrichous flagella
F-9	Human blood	Friewer, Ill. Pub. Health No. 381BC. Routine blood culture
F-16	Human stool	Friewer, Ill. Pub. Health No. 758A. Typhoid carrier
F-32	Human stool	Friewer, Ill. Pub. Health No. 1505RZ. Typhoid carrier
F-36	Human blood	Friewer, Ill. Pub. Health No. 2398BC. Typhoid convalescent
F-76	Human stool	Friewer, Ill. Pub. Health No. 3179A. Typhoid patient
F-79	Human stool	Friewer, Ill. Pub. Health No. 2869A. Typhoid convalescent
F-101	Human stool	Friewer, Ill. Pub. Health No. 1575R1. Routine culture
F-112	Human stool	Friewer, Ill. Pub. Health No. 2068A. Typhoid convalescent
F-125	Human blood	Friewer, Ill. Pub. Health No. 270BC1. Routine culture
K-1	Human stool	Kennedy, Cook County Hosp. Routine stool culture
G-1	Water	Galarneault. Stagnant water in jar with plants
G-2	Water	Galarneault. Chicago sewage
G-3	Human stool	Galarneault. Routine stool culture
G-4	Human abdomen	Fulton No. 2781. Abdominal swab

TABLE II

MEAN STRAIN WAVE LENGTHS (W.L.) AND AMPLITUDES, AND THEIR STANDARD DEVIATIONS (S.D.)

Strain No.	W.L.	S.D.	Amp.	S.D.	
H-17	3.18	0.32	1.11	0.09	
H-31	3.13	0.23	1.12	0.12	$\dfrac{\text{Polar W.L.}}{\text{Polar Amp.}} = \dfrac{3.10}{1.08} = 2.87$
H-37	3.20	0.28	1.11	0.11	
H-131	3.20	0.25	1.10	0.09	
H-133	3.01	0.31	1.15	0.09	$\dfrac{\text{Polar W.L.}}{\text{Nonpolar W.L.}} = \dfrac{3.10}{1.05} = 2.95$
H-226	3.08	0.17	1.01	0.09	
H-261	3.10	0.24	1.01	0.13	
F-16	2.98	0.28	1.09	0.11	$\dfrac{\text{Nonpolar W.L.}}{\text{Nonpolar Amp.}} = \dfrac{1.05}{0.42} = 2.50$
F-36	3.08	0.17	1.04	0.12	
G-3	3.07	0.22	1.13	0.11	
Species mean	3.10	0.08*	1.08	0.04*	
H-260	1.05	0.09	0.42	0.04	

*Standard deviation of strain means from species mean.

Experimental Results

Morphology

All cultures of the group were, by primary selection, Gram-negative rods with tufts of polar flagella. In some cultures the rods were slightly curved, in others quite straight. The usual size of the soma was 0.5–1 μ by 2–3 μ. The polar flagella have a very characteristic shape, unlike those of most flagellated bacteria, but very similar to those of the spirilla (see Leifson (8) and the illustrations in this paper). The flagella generally have one curve and rarely more than two curves. The most common number of flagella at one pole is two to four. In some strains the organisms are mainly unipolar, in others bipolar (Figs. 1–6).

The wave length and amplitude of 10 separate flagella were measured for each of 10 selected strains, and for the peritrichous variant, H-260. In many strains, most of the flagella showed less than one complete wave and the wave length was determined from the measurements of one-half wave. In Table II are recorded the means of these measurements and the standard deviations of the individual measurements from the respective strain means.

Photomicrographs, magnification 2100 ×. Leifson flagella stain.

FIGS. 1–5. Typical examples of the flagellation of *Lophomonas* species.

FIG. 5 illustrates a fairly common type in which the soma does not take the flagella stain.

FIG. 6. Filamentous variant of *Lophomonas* strain H-261.

FIGS. 7, 8, and 9. Intermediary stages in the mutations of H-261 to the peritrichously flagellated H-260. Note the difference in wave lengths of the polar and nonpolar flagella.

FIG. 10. An example of the peritrichous variant (H-260) of strain H-261. The number of flagella per organism varied from 1 to 6. Strain H-260 is a typical *Alcaligenes* species.

FIGS. 11, 12, and 13. *Spirillum virginianum* showing a type of flagellum very similar to those of *Lophomonas*. The single individual shown in Fig. 11 is very similar to *Lophomonas*. Filaments of *Lophomonas* (Fig. 6) however do not form a spiral and are quite different from a typical *Spirillum* as illustrated in Fig. 13.

PLATE I
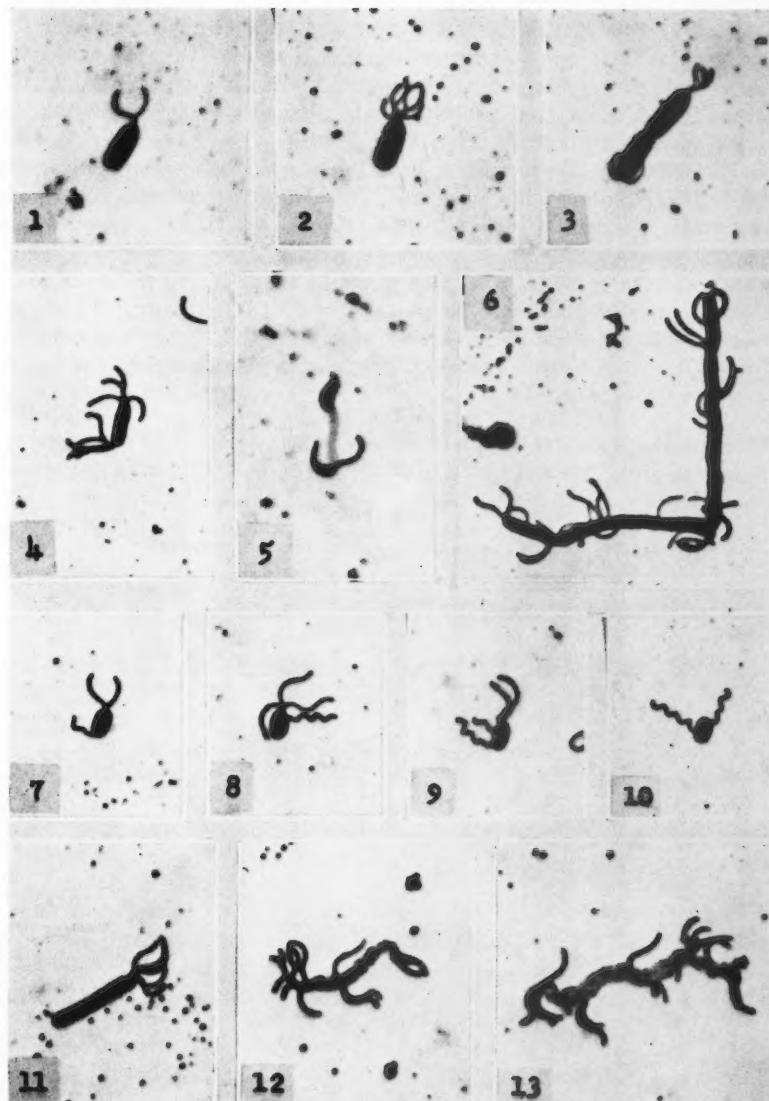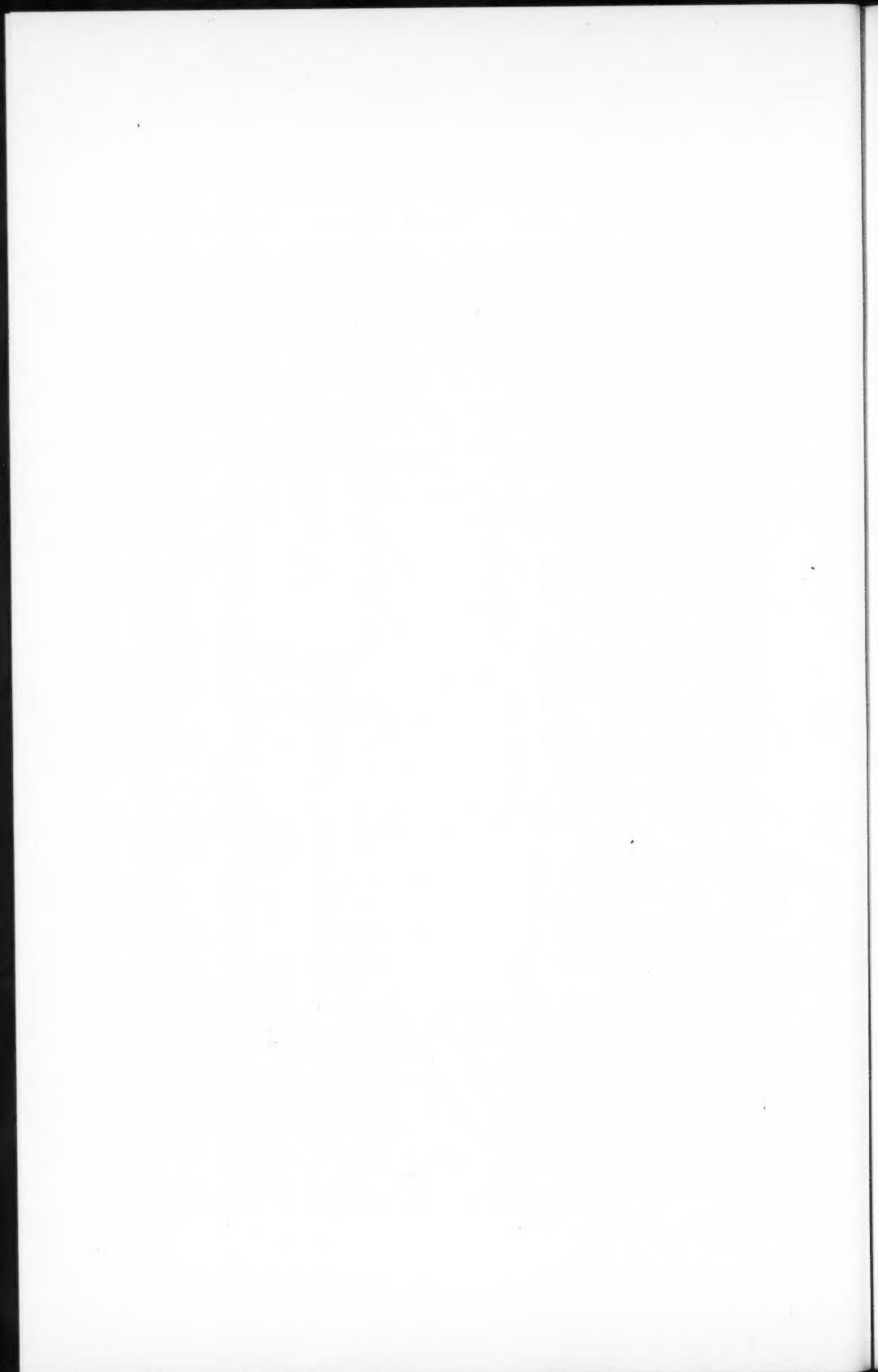
Statistical analysis of variance showed the strain mean differences to be insignificant. We may therefore regard all the strains as having polar flagella of essentially the same shape.

The nonpolar flagella developing at a rather high frequency in the genetically unstable strain, H-261, are strikingly different from the polar flagella (Figs. 7–10). Each flagellum usually has several curves with an average wave length of slightly over one micron, or about one-third that of the polar flagella. Individual organisms showing both types are transitional types which segregate into pure polar and pure nonpolar flagellar types. Mutation of the peritrichously flagellated strain H-260 to a polarly flagellated type has not been observed. This is in agreement with the observations of Leifson and Hugh (9).

Strain H-261 is the only one of the 25 strains studied which has shown visible flagellar mutation. It is interesting to note that the parent strain (H-247) has been on artificial media for over 30 years while the other strains are not over 10 years old and most are fairly recent isolates.

Physiology

The organisms have the general physiological characteristics associated with *Alcaligenes* sp. (Table III). Carbohydrates are not attacked either fermenta-

TABLE III

PHYSIOLOGICAL REACTIONS OF *Lophomonas* STRAINS

Substrate	H-17, 31, 32, 37, 124, 131, 133, 226; F-16, 36, 76, 79, 101, 112; K-1; G-2	H-185	H-212	H-261	G-4	F-9 F-125	F-32	G-1	G-3
Dextrose, lactose, sucrose, maltose, mannose, arabinose, xylose	—	—	—	—	—	—	—	—	—
Methanol	—	+	?	+	—	?	—	—	?
Ethanol	—	?	+	—	—	+	+	—	?
Ethylene glycol	—	+	+	—	—	+	+	—	+
Glycerol	+	+	+	+	+	+	+	+	+
Erythritol	—	+	+	—	—	+	+	—	+
Adonitol	—	?	+	—	—	+	+	—	+
Mannitol	—	+	+	—	+	+	+	+	+
Sorbitol	—	?	+	—	+	+	+	—	?
Dulcitol	—	?	+	—	—	+	+	—	?
Inositol	—	?	—	?	—	?	+	—	?
Indole, M.R., V.P., gelatin, coagulated serum, urea, blood hemolysis	—	—	—	—	—	—	—	—	—
Litmus milk	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
Nitrate, oxidase, catalase, hydrogen sulphide	+	+	+	+	+	+	+	+	+
Simmons citrate agar	—	+	—	—	—	+	+	—	—
Anaerobic growth with nitrate	+	+	+	—	—	+	+	+	+
Anaerobic growth without nitrate	—	—	—	—	—	—	—	—	—
Growth on desoxycholate agar	+	+	+	+	+	+	+	+	+

+ *means acid with carbohydrates and alcohols, and the usual meaning with other reactions.*
? *means doubtful reaction.*
— *means negative reaction.*

tively or oxidatively and peptone media become alkaline. All strains oxidized glycerol more or less promptly. The action on the other alcohols was variable and, where positive, the acid produced was small. Nitrate was reduced to nitrite by all strains. With two exceptions, all strains grew anaerobically in the presence of nitrate but not without nitrate. This is contrary to our experience with most nonfermentative bacteria (except those which reduce nitrate to nitrogen gas) which cannot grow anaerobically either with or without nitrate. The reaction on Simmons citrate agar was variable but never strongly positive and seems to be of minor taxonomic importance. Hydrogen sulphide was produced in peptone media by all strains and could be detected by lead acetate either on paper strips or incorporated into the medium. With iron salts hydrogen sulphide production was not detectable. All strains turned litmus milk blue, without coagulation or clearing. All strains gave a positive oxidase test. Since this test is negative for many types of bacteria it may have taxonomic importance in identifying the group. Catalase was also produced by all strains but this is very common among bacteria. All other tests were negative.

Antibiotic Sensitivity

Some variation was found among the different strains. Greatest sensitivity was found to aureomycin, chloromycetin, and terramycin; somewhat less to dihydrostreptomycin and streptomycin. The following had little growth inhibitory effect: penicillin, bacitracin, sulphathiazole, sulphadiazine, and polymyxin B.

Antigenicity

Flagellar and somatic agglutination revealed considerable antigenic hetero-geneity in the group. In most instances the H and O agglutinations corresponded perfectly. In Table IV are recorded the serological reactions to selected sera. It may be seen from the table that the strains fall into six groups of two or more with a number of strains which are unrelated to the others. The seven strains of the largest group appear to be practically identical. Six of these strains were from human stools and one from Chicago sewage. All of these strains were physiologically alike.

Source

The source of our strains is given in Table I. Fifteen strains were obtained from human stools, four strains from human blood, and six strains from other sources. We have no definite indication that any of the strains had been the cause of human disease.

Historical

A survey of the literature shows that organisms similar in morphology and physiology to our group have been isolated quite frequently and from a variety of sources. The organisms appear to be the most common hetero-trophic Gram-negative rods, which do not attack carbohydrates, found in the

TABLE IV

H AND O AGGLUTINATION TITERS WITH SELECTED ANTISERA

Antigen		H-32	H-37	H-124	H-131	H-185	H-261	F-36	K-1
H-17	H				5120				
	O				1280				
H-32	H				5120				
	O				640				
H-131	H				5120				
	O				1280				
H-133	H				5120				
	O				1280				
F-79	H				5120				
	O				2560				
F-112	H				5120				
	O				640				
G-2	H				2560				
	O				1280				
F-16	H							40	640
	O							—	320
F-36	H							80	1280
	O							—	160
K-1	H								5120
	O								1280
H-185	H					5120			
	O					1280			
G-1	H					640			
	O					160			
G-3	H					1280			
	O					320			
H-37	H		2140						
	O		640						
F-101	H		2560						
	O		320						
H-31	H	10240		80					
	O	1280		40					
H-124	H	640		10240					
	O	160		2560					
F-9	H	80		40			80		
	O	—		—			40		
F-125	H	160		40			80		
	O	—		—			40		
H-226	H						80		
	O						40		
H-261	H						1280		
	O						320		
F-32	H							640	
	O							320	

human intestine.   Since they grow well on bile salt media, such as desoxycho-late agar, they are occasionally found on stool culture plates, and more often than not labelled *Alcaligenes*.

Gunther (3) isolated from soil and described a vibrio-shaped organism with a tuft of polar flagella.   This appears to be the first reported isolation of our organism.   Petruschky (10) introduced the term *Bacillus fecalis alcaligenes* for organisms he had isolated from human stools.   Although Petruschky stated that his organisms had peritrichous flagella the epithet *alcaligenes* became generally applied to any non-fastidious, heterotrophic Gram-negative rod which had no effect on carbohydrates.   A rather lively controversy took place in Germany between the years 1900 and 1917 regarding the flagellation of *B. fecalis alcaligenes*.   The most common, and in some instances the only, organism isolated from human stools with the alcaligenes physiology had lophotrichous flagella (e.g. see Conradi (2) and Kuhnemann (6)).   This led many bacteriologists to feel that Petruschky probably was mistaken in his report on flagellation (he gave no photographic proof).   Lehmann and Neumann (7) proposed the name *Vibrio alcaligenes* for the organism previously labelled *B. fecalis alcaligenes* or *Bacterium alcaligenes*.   They apparently had been led to believe that all "alcaligenes" types possessed lophotrichous flagella and a slightly curved soma.   This proposal by Lehmann and Neumann does not appear to have received widespread adoption.

A review of the literature dealing with the lophotrichous bacteria is difficult to write since only a few papers are found in which the flagellar morphology is stated.   Lophotrichous organisms were isolated from human stools by Conradi (2); from the intestine of cattle by Horn and Huber (4); from human stools by Baerthlein (1), by Pollack (11), and by Stitzer (13); from human abscess by Severi (12); from human stools, and from water by Türck (14).   Various reports in the literature on the pathogenicity of *Alcaligenes* may have dealt with the lophotrichous type, but since flagellation was not given we can only guess.

Discussion and Taxonomy

The 25 strains of lophotrichous bacteria that we have described seem sufficiently alike to form a single species.   The flagellation as well as the soma of all strains is very similar.   Physiologically the strains are basically alike.   Of greatest taxonomic importance is the uniformly negative reaction on carbohydrates and gelatin, and the uniformly positive reaction with nitrate, catalase, hydrogen sulphide, and glycerol.   Some strains metabolize, to a slight extent, a number of alcohols and citrate but these weak reactions do not seem taxonomically significant.   The variation found in antigenic constitution is not generally considered taxonomically significant at the species level.   For the group we suggest the species designation *alcaligenes*.

For a genus we have several possibilities.   Lehmann and Neumann (7) suggested *Vibrio*.   We are not in agreement with this suggestion.   The type species of the genus *Vibrio* is a polar monotrichous flagellated rod with a

somatic curvature; it ferments carbohydrates and is actively proteolytic. Our lophotrichous organisms have none of these characteristics except for a very slight somatic curvature shown by some strains. Two other genera may be considered, namely *Pseudomonas* and *Spirillum*. The flagellation of typical species of *Spirillum* (and *Rhodos pirillum*) is very similar to that of our group. The soma of spirilla tends to be much more curved. We have in our collection, however, a culture named *Spirillum virginianum* in which the somatic unit has only one curve as a rule and this not very pronounced (Figs. 11–13). Physiologically the spirilla and our group are alike in that neither attack carbohydrates, but differ in that spirilla are typically gelatin liquefiers and nitrate negative while our group is gelatin negative and nitrate positive. The genus *Pseudomonas* at present seems greatly overextended. The type species, *Pseudomonas aeruginosa*, is a straight rod with a polar monotrichous flagellum. Physiologically the organism oxidizes a number of carbohydrates, produces a greenish water soluble pigment, and reduces nitrate to nitrogen gas. Our group does none of these. Included in the present *Pseudomonas* genus however are species which resemble our group much more closely than does the type species, such as monotrichous rods which are nonpigmented, reduce nitrate to nitrite only, and do not oxidize carbohydrates; and many species of plant pathogens with polar multitrichous flagella, no pigment, and nitrate reduced to nitrite only. We feel it would be a mistake to further extend the *Pseudomonas* genus by including in it the lophotrichous organisms. The best alternative appears to be to create a new genus for our lophotrichous bacteria.

The observation by Leifson and Hugh (9) that a strain of lophotrichous bacteria (ATCC No. 8461) spontaneously mutated from lophotrichous flagellation to peritrichous flagellation has been a difficult problem to deal with taxonomically. None of our other strains has shown this phenomenon. The peritrichous variant, H-260, is a typical *Alcaligenes* species. Does this indicate a close relationship between the lophotrichous group and the peritrichously flagellated *Alcaligenes*? Perhaps, but in the interest of a practical working taxonomy the two flagellar types are better classified into separate genera. We suggest the generic name *Lophomonas* for our group, specifically **Lophomonas** *alcaligenes* n. gen. This name may be considered a synonym of *Vibrio alcaligenes* Lehmann and Neumann.

CHARACTERISTICS OF THE GENUS *Lophomonas*

MORPHOLOGY: Rod-shaped, occasionally slightly curved soma, 0.5 to 1.0 by 2 to 3 μ. Filaments may be present in some cultures. Motile, Gram-negative. Flagellation polar, lophotrichous, with an average of 2–4 flagella per organism. Flagella have typically fewer than two curves with an average wave length of 3.1 μ and average amplitude of 1.08 μ. Twenty-four of the 25 strains studied have shown no evidence of morphological instability. One strain studied is genetically unstable and mutates through a series of intermediary stages to a stable peritrichously flagellated type. Reverse mutation has not been observed.

CULTURAL CHARACTERISTICS: Colonies on agar are smooth, edges entire, semitranslucent. Growth on agar slants is moderate, smooth, colorless, and semitranslucent. Growth in agar stab confined mainly to the surface. Growth in broth is turbid with pellicle formation. No growth in nutrient broth under strictly anaerobic conditions unless nitrate is present. Temperature relations, mesophilic; pH relations, neutrophilic; oxygen relations, aerobic; pigmentation, none.

PHYSIOLOGICAL CHARACTERISTICS: Carbohydrates are not attacked (acid is not produced nor does the carbohydrate disappear) including glucose, lactose, sucrose, maltose, mannose, arabinose, and xylose.   Glycerol is oxidized but other alcohols variable.   The following tests are uniformly negative: indole, M.R., V.P., gelatin, coagulated serum, urea, blood hemolysis. The following tests are uniformly positive: nitrite, oxidase, catalase, and H_2S.   Simmons citrate agar variable.   Growth on desoxycholate agar uniformly good.   Litmus milk turns blue without coagulation or clearing.

HABITAT: Common in human intestine, also found in water, soil, etc.

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GENERAL CHARACTERISTICS OF CERTAIN INSECT PATHOGENS RELATED TO BACILLUS CEREUS[1]

BY T. A. ANGUS[2]

Abstract

The insect pathogens *Bacillus sotto* Ishiwata and *Bacillus thuringiensis* Berliner can be differentiated by means of certain cultural and morphological differences, and by a quantitative difference in pathogenicity for the larvae of *Bombyx mori* L., *B. sotto* being the more pathogenic. They are also pathogenic for the larvae of several North American Lepidoptera including *Anisota rubicunda* (F.), *Anisota senatoria* (A. and S.), *Nymphalis antiopa* (L.), *Erannis tiliaria* (Harr.), *Datana integerrima* (G. and R.), *Datana ministra* (Drury), *Liparis dispar* L., *Protoparce quinquemaculata* (Haw.), and *Protoparce sexta* (Johan.). Silkworm larvae ingesting material from a sporulated culture of *B. sotto* become sluggish, cease feeding, and suffer from a progressive paralysis that begins in the mid-gut area, spreads to affect the whole larva, and ends in death.

Introduction

Although the bacterial pathogens of insects belong to several genera, the best known are species of the genus *Bacillus* (8, 20). Recent work in Europe and North America has focussed attention on several species, closely related to *B. cereus* Frankland and Frankland, which are pathogenic for lepidopterous larvae. These include *B. sotto* Ishiwata (12) and *B. cereus* var. *alesti* Toumanoff and Vago (28) which are pathogens of the silkworm *Bombyx mori* L., and *B. thuringiensis* Berliner a pathogen of the flour moth, *Ephestia kühniella* Zell. (20).

In 1953 during a study of sporulation in *B. thuringiensis*, Hannay (9) noted that the spores were invariably accompanied by what appeared to be diamond-shaped crystals. Since the crystalline inclusions were present only in strains known to be pathogenic for insects, Hannay suggested that the crystals might be "in some way connected with the formation of a toxic substance encouraging septicaemia of insect larvae."

In a preliminary note in 1954 Angus (2) presented evidence to show that the toxic action of *B. sotto* is due to a toxin as suggested by Aoki and Chigasaki (4) and that the toxic material is associated with crystalline inclusions as suggested by Hannay (9). Here and in subsequent papers this evidence will be given in detail, and as well some additional information regarding the toxicity of *B. sotto*.

B. sotto was studied by Aoki and Chigasaki and in a series of papers in 1915 and 1916 (3, 4, 5, 6) they described the organism and its paralyzing effect on silkworm larvae. They suggested that the lethal action of *B. sotto* was not due to the growth of the microorganism in affected insects but rather to the action of some toxic substance elaborated during the growth of the bacteria

on artificial culture media. Toumanoff and Vago (30) reported similar results with *B. cereus* var. *alesti* and ascribed death to toxemia, septicemia, or intermediate conditions.   Steinhaus (22) described symptoms in the larvae of *Colias philodice eurytheme* Boisd. (the alfalfa caterpillar) fed spores of *B. thuringiensis* as "cessation of feeding, sluggishness, diarrhea and regurgitation, and finally death".   Similar symptoms have been observed in other insects (15, 24).

Some investigators (10, 23, 31) have found that young cells of *B. cereus* strains when ingested by lepidopterous larvae may, under certain conditions, cause death of the insects.   There is no paralysis of the larvae and death does not usually occur under 24 hr. and more often not until 36 hr. after ingestion of the bacterial suspension.   Cells of the organism fed are sometimes found in the body cavity of affected insects.   This is the condition described by Steinhaus (23) and Tanada (24) and called "septicemia" by Toumanoff and Vago (30).

In contrast to septicemia, which is a slow process, is the rapid paralysis that occurs usually within two to six hours after ingestion of a suspension of a sporulated culture of certain species of bacteria.   This condition has been called "toxemia" by Toumanoff and Vago (30).

B. sotto, *B. thuringiensis*, and *B. cereus* var. *alesti* are very similar and this has been discussed by Toumanoff (26) who noted that the *alesti* strain alone produces a red pigment.   The criteria for separating *B. sotto* and *B. thuringiensis* are not so clear-cut.   In the present paper certain consistent cultural and morphological differences between *B. sotto* and *B. thuringiensis* are described. In addition, the pathogenicity of these strains for lepidopterous larvae is compared.

Materials and Methods

Sources of Cultures

The control or reference organism in these studies was *B. cereus* Frankland and Frankland (No. 9818 in the American Type Culture Collection) which is not pathogenic for lepidopterous larvae.   The culture of *B. sotto* used was obtained through Mr. M. Ono of the Sericultural Experiment Station in Tokyo.   Professor E. A. Steinhaus supplied the culture of *B. thuringiensis* used and it is referred to, by him, as "Mattes' " strain (15, 23).   Dr. S. E. Jacobs of the Imperial College of Science and Technology, London, supplied cultures of the three strains that he isolated from "Sporeine" (13).   These are designated, using Jacobs' coding, as Sporeine 4X, Sporeine I, and Sporeine II.

Cultural Methods

When a new culture was received it was subcultured to a single tube of broth and from that to 40 slants of nutrient agar, a number of which were checked for purity.   These stock slants were used as the source of inoculum for all studies.   By using a secondary transfer only once and conducting all tests with material only three transfers removed from the original culture, the risk of cross-contamination was lessened.

Many strains of *B. cereus* have been studied by Smith, Gordon, and Clark (19) and by Knight and Proom (14).   These authors are agreed that a number of characteristics are of importance in identifying *B. cereus*.   Tests to determine these characteristics and others were made on *B. sotto* and other insect pathogens.   In general, the media were prepared using the formulations given by Smith, Gordon, and Clark (19).   The cultures were incubated at 28° C.   For routine culturing Difco nutrient agar and broth were used.

Voluntary Ingestion Tests

The most satisfactory method of testing the pathogenicity of various preparations was by inducing larvae to feed on leaves to which a suspension containing the material to be tested had been applied.   The test organism was grown on or in a suitable medium, harvested, and resuspended.   When the cell count had been established, the suspension was diluted as required and applied to a leaf surface in measured amounts.   The cells were harvested and suspended in water.   The number of cells in a suspension was determined by plate counts or by turbidimetric measurements (25).

One of the problems encountered in feeding tests of insect pathogens was wilting of the foliage.   To reduce this, the drying time was shortened by spreading the test dose over the whole leaf surface and drying in a stream of air.   For small mulberry leaves (2 sq. in.) 0.05 to 0.1 ml. was found to be optimal; the commercial sticker Methocel* was used to facilitate spreading (1).   Whole leaves, coated and dried while still attached to the parent plant were sometimes used.   When testing small larvae it was sometimes necessary to permit a number of larvae, never exceeding 10, to feed on a single treated leaf; it was assumed that uniformly sized larvae starved for the same period ingest equal amounts of foliage.

Forced Ingestion Tests

The method described above has been called voluntary ingestion.   In tests with certain insect species it was necessary to resort to forced feeding and a suction holder similar to that described by Heimpel (11) was used.   A dropping pipette, a loop, or a probe was used to introduce a dose of the material being tested into the buccal cavity of larvae.

Injection Tests

In some experiments, the material being tested was injected into the body cavity of larvae through an abdominal proleg with a microsyringe.   All toxicity tests mentioned were feeding tests unless otherwise stated.

Experimental Insects

The principal insect used in these studies was a laboratory strain of the silkworm, *Bombyx mori* L.   In winter, larvae are fed on mulberry foliage grown in a greenhouse, in summer on foliage obtained from southern Ontario.

Dow Chemical Company of Canada, Ltd., kindly supplied experimental quantities of Methocel.

The supply of mulberry foliage is limited during the winter months, and as a consequence some of the experiments in these studies are based on small numbers of insects.

Observations and Results

CULTURAL STUDIES

All the strains tested, *B. cereus* No. 9818, *B. sotto*, *B. thuringiensis*, and *Bacillus* spp. I, II, 4X isolated from Sporeine powder, gave practically identical results in the biochemical tests: abundant growth on nutrient agar at pH 7.5 and moderate growth at pH 6.0; producing acid alone from glucose but not from arabinose or xylose; producing acetylmethylcarbinol; hydrolyzing starch, casein, and gelatin; utilizing citrate; able to grow anaerobically; producing lecithinase as shown by a positive egg-yolk reaction (16). All belong to Morphological Group I of Smith, Gordon, and Clark (19) and to the amino-acids-requiring group of Knight and Proom (14) with respect to nitrogen source and essential metabolites.

Only *B. sotto*, *B. thuringiensis*, and *Bacillus* spp. I and 4X produced crystal-line inclusions (9). They also caused paralysis in silkworm larvae whereas *B. cereus* No. 9818 and *Bacillus* II were not pathogenic.

B. sotto and *B. thuringiensis* could not be differentiated by biochemical tests and were almost identical culturally except for consistent and easily recognized differences in pellicle formation and cell size. *B. thuringiensis*, in nutrient broth, always forms a firm pellicle which does not disperse but sinks to the bottom when the tube is gently tilted. More violent shaking of the tube will break the pellicle into relatively large flakes which persist. *B. sotto* under similar conditions does not usually produce a pellicle and if it does, this occurs long after the *B. thuringiensis* pellicle appears. The *B. sotto* pellicle is very soft and only gentle tilting of the culture tube is required to disperse it into a diffuse homogeneous suspension. Toumanoff (26, 27) has also noted differences in pellicle formation.

A difference was seen also in cultures grown on nutrient agar. Plates of nutrient agar were inoculated by coating the agar with 1 ml. of an 18-hr. old culture of *B. sotto* or *B. thuringiensis*. After incubation for 100 hr. at 28° C., 5 ml. of sterile water was gently pipetted over the bacterial growth. After a few minutes, gentle tilting of the Petri dish freed the growth from the agar surface. *B. sotto* growth dispersed into a cloudy, homogeneous suspension immediately. *B. thuringiensis* growth broke up into rather large flakes that did not dissipate on standing. This difference could be demonstrated in water suspensions that had been stored at 3° C. for as long as three months.

Plate I shows differences in the morphology of vegetative rods of *B. sotto* and *B. thuringiensis* grown under similar conditions. It will be noted that the vegetative rods of *B. sotto* tend to be somewhat longer and thinner than those of *B. thuringiensis*. The difference is obvious in all growth stages until the vegetative cells begin to shorten and thicken to form sporangia.

PLATE I
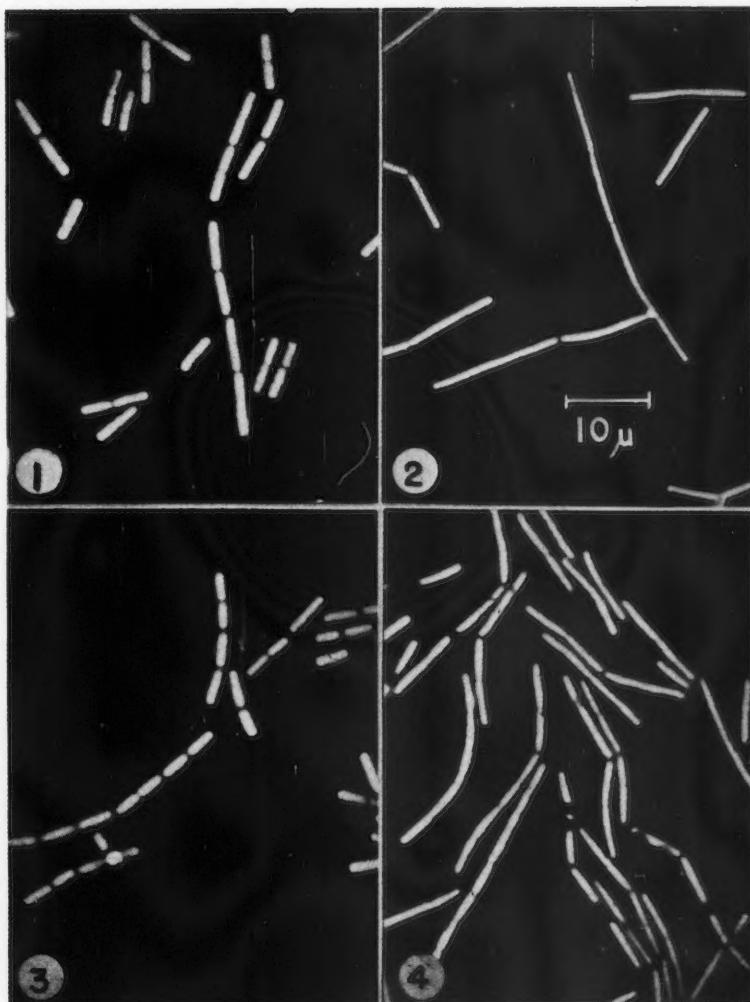
Vegetative cells of *B. thuringiensis* and *B. sotto* grown on nutrient agar at 25° C.
 FIG. 1. *B. thuringiensis* after 12 hr.
  FIG. 2. *B. sotto* after 12 hr.
  FIG. 3. *B. thuringiensis* after 30 hr.
  FIG. 4. *B. sotto* after 30 hr.
Nigrosin air-mounted films (Robinow's technique).
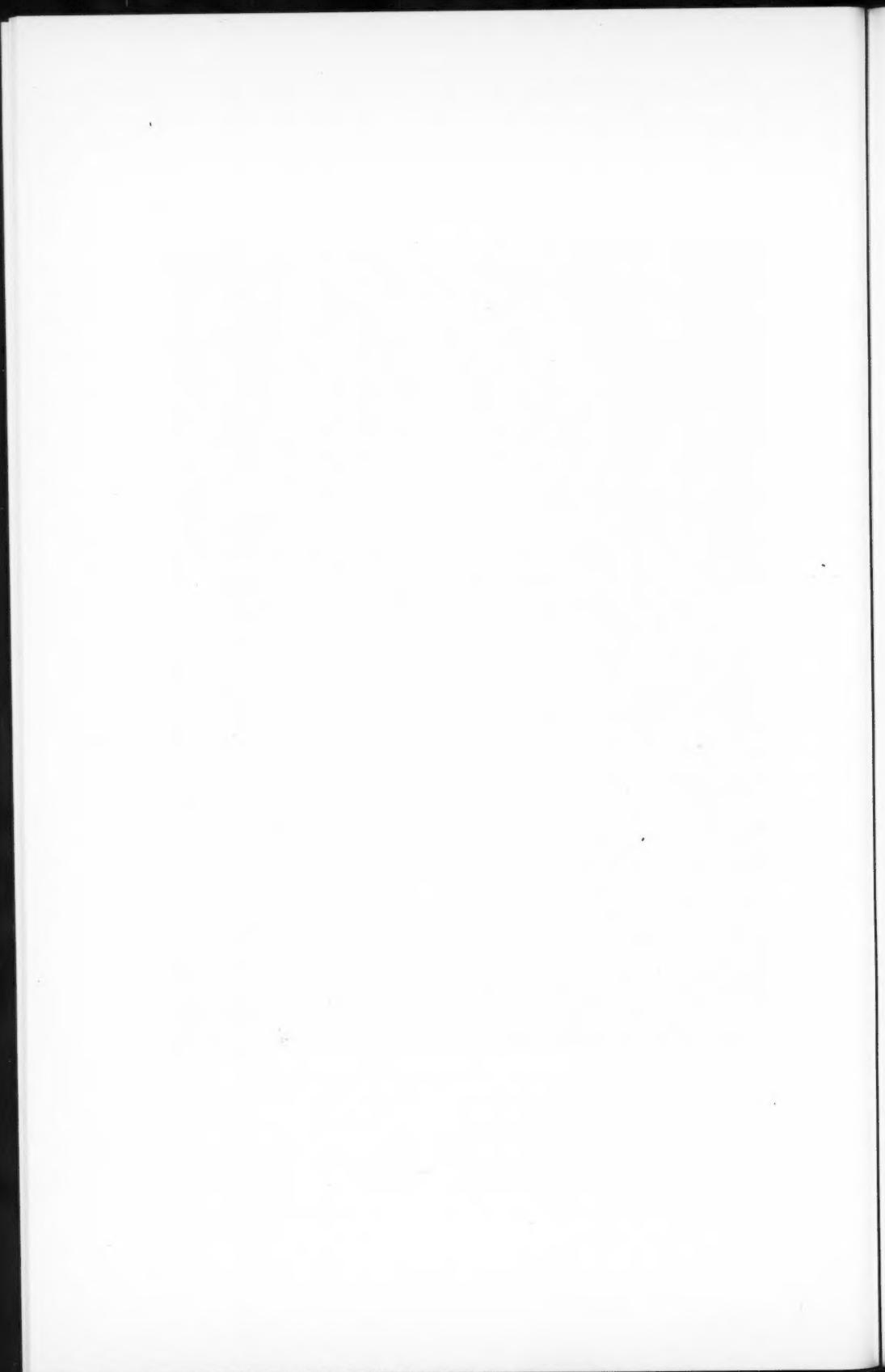
The most striking difference between *B. sotto* and *B. thuringiensis* is in their relative pathogenicity for silkworm and other larvae (7, 17, 26, 29). This difference is expressed in quantitative terms below.

PATHOGENICITY STUDIES

Symptoms in Silkworm Larvae

A number of responses and abilities were studied in order to determine the nature of the paralysis observed in silkworm larvae. Larvae, all reared from the same egg mass and presumably genetically similar, were divided into two groups. The control group was allowed to feed on foliage coated with a dilute, heat-killed (225° C. for 30 min.) suspension of a sporulated *B. sotto* culture. The other group of larvae was allowed to feed on foliage that had been coated with a dilute unheated suspension of a sporulated *B. sotto* culture. These larvae became paralyzed and eventually died. The changes noted are summarized in Table I which shows that there was a sequence of symptoms in affected silkworm larvae. The first symptom was cessation of feeding and sluggishness of the larvae. This was difficult to measure other than by comparing the amount of food eaten by the two groups of larvae. By weight, it was found that the control larvae had eaten from two to three times as much in the same time as had larvae of the other group.

The second symptom was less obvious and may be described as a slight distension of the body wall in the abdominal region. If a normal silkworm larva is rolled over, it immediately curls into a crescent from which it twists into the upright position. In affected larvae, shortly after the abdominal swelling was noted, the ability to curl was lost and the insects were unable to right themselves. This, the so-called "sotto position" (4), is quite characteristic. The abdominal prolegs then became affected and were no longer able to clasp the edge of a leaf or of a sheet of paper thrust between them. Following this, the symptoms spread towards the anterior and posterior ends simultaneously. The anal prolegs and thoracic legs were affected at about the same time. The mandibles were the last external structures visibly responding to stimuli.

Several interesting changes were noted in the general muscular tone. In the first stages of paralysis, as noted before, a slight distension was noted in the abdominal region and the larva became semirigid in this area. As the symptoms spread so did the rigidity until the whole insect became comparatively stiff. This rigidity soon gave way to a flaccid condition, in which the larva became completely limp.

In a normal mature silkworm larva, the rhythmic pulsation of the heart is visible through the dorsal integument. In the early stages of paralysis, the heartbeat was still strong and regular. As the paralysis spread, the beat became weaker and irregular. However, it persisted even after the insect had passed from the rigid into the flaccid condition.

Larvae were dissected just before and immediately after paralysis was observed and, apart from a slight distension of the gut, no consistent macro-

TABLE I

DEVELOPMENT OF PARALYSIS IN SILKWORM LARVAE INFECTED WITH *B. sotto*

Normal larvae	Changes noted in infected insects at				
	90 min.	105 min	135 min.	165 min.	180 min.
Mandibles move in response to stimuli	Mandibles move normally	Mandibles move normally	Mandibles flutter continuously	Only discernible movement is fluttering of mandibles	Insect flaccid
All legs move rationally; move to clasp on paper or leaf edge	Insect still able to clasp	Only anal prolegs and thoracic legs able to clasp	Thoracic legs flutter continuously. Neither anal prolegs nor thoracic legs can clasp		
Heartbeat regular	Heart still beats	Heart still beats	Heartbeat irregular		
Lifts head and thoracic region in response to stimuli	Body of insect semirigid	No response to stimuli	Insect rigid		
When rolled on back or side of body, rights self quickly and easily	Cannot right self				
Easily orientates self while clasping slowly twirled rod	Hold less firm	No longer able to clasp			

scopic changes were visible.   The viscosity and color of the gut contents and
of blood extracted from the hemocoele were unchanged.

An oral or anal discharge sometimes occurred but only after the insect had
been given massive doses of the test suspension.   It was unlike the diarrhea
that occurs in other insects following ingestion of certain bacterial suspensions.

The term "melanosis" has been applied (21) to the blackening that occurs
in insects following death from bacterial infection.   Normal silkworm larvae
are light in color, varying from gray-white to light cream.   Consequently,
the beginning of melanosis is easily seen.   In larvae that had ingested *B. sotto*,
melanosis began about 8 to 10 hr. after the onset of paralysis as a chocolate-
brown or black spot in the abdominal region and slowly spread along the
entire length of the body.

Symptoms in Other Insect Species

No detailed study was made of the symptoms in the larvae of the other
insect species tested.   In general, the symptoms were similar to those seen by
Steinhaus (22) and by Tanada (24) in other Lepidoptera.   Paralysis in six
hours was rare; sluggishness followed by diarrhea was more common.   The
larvae died between 12 and 100 hr. after starting to feed.

Comparative Pathogenicity of B. sotto and B. thuringiensis for Silkworm Larvae

Tests were made to determine the LD_{50} of a particular bacterial preparation
for *B. mori* larvae.   A simplified probit-analysis was used (Schelling (18)),
and the results obtained are expressed in terms of viable cells per dose.

Using fourth- and fifth-instar silkworm larvae as test insects, the *B. thu-
ringiensis* LD_{50} dose was 1,000,000–1,500,000 viable cells per larvae; for
B. sotto the LD_{50} dose was much less, 40,000–50,000.   The results of a typical
test are illustrated in Fig. 5.   Although the LD_{50} values are expressed in
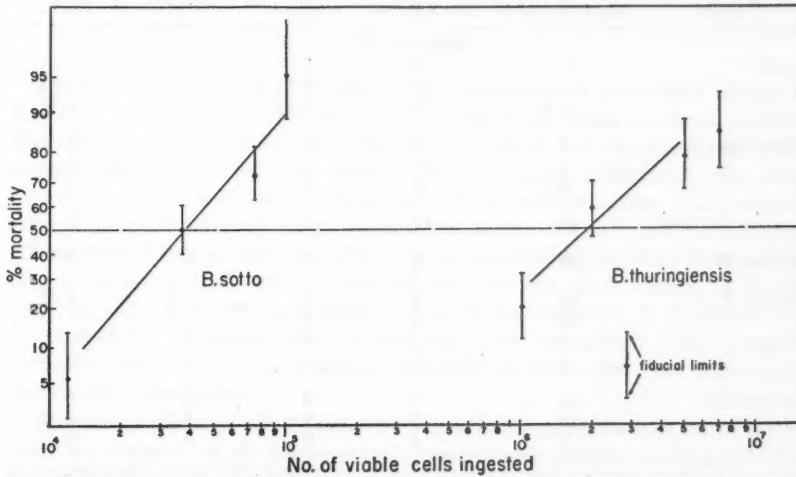

FIG. 5.   Comparative pathogenicity of suspensions of sporulated cultures of *B. sotto*
and *B. thuringiensis* for *Bombyx mori* larvae.

terms of the number of viable cells per dose, it was appreciated that such values are significant only if mortality depends on activity of living cells. When later it was found that this is not the case and that germination and multiplication of ingested material are not responsible for the observed mortality, LD_{50} values were based on the weight of dried cells (viable and non-viable) ingested.

The various strains tested were grown on nutrient agar for 150 hr. at 28° C., harvested separately in water, centrifuged, and the pellet of washed spores was dried in high vacuum over silica gel.   The dried spores were resuspended in water to give 30 mgm. of dried material per milliliter of suspension.   The suspensions were diluted serially and 0.05 ml. of each suspension was spread on a leaf, air-dried, and fed to larvae.   The results of two such tests are given in Table II and show that *B. sotto* was more toxic than *B. thuringiensis*.   With all dilutions no larvae were paralyzed within 75 min. after starting to feed; after this period the onset of paralysis varied with the dosage.   With very dilute suspensions, paralysis sometimes did not occur until after eight hours. Tests with toxic extracts, to be discussed in a subsequent paper, showed that the lethal dose is directly proportional to the weight of the larva.

Toumanoff and Vago (28) noted that starved silkworm larvae were more susceptible than fully fed larvae to *B. cereus* var. *alesti*.   Tests showed that this was also the case for *B. sotto*.

TABLE II

PATHOGENICITY OF CERTAIN BACTERIAL STRAINS FOR SILKWORM LARVAE*

Culture	Dried cells, μgm. per larva	No. of larvae	Paralysis (P) or death (D) in		
			4 hr.	17 hr.	48 hr.
B. sotto	2	5	3 P	5 P	5 D
	1	16	10 P	15 P	15 D
	0.4	10	2 P	7 P	7 D
B. thuringiensis	2	5	0	0	0
	1	16	0	0	0
	0.4	10	0	0	0
B. cereus No. 9818	30	15	0	0	0
Sporeine II	30	5	0	0	0
Sporeine I	30	5	0	2 P	4 D
	15	5	0	1 P	1 D
	3	5	0	0	0
	1.5	5	0	0	0
Sporeine 4X	30	5	0	2 P	4 D
	15	5	0	1 P	1 D
	3	5	0	0	0

*Laboratory-reared white strain; average weight about 200 mgm.; fourth instar.

Tests with Other Insect Species

The susceptibility of other larvae to infection with *B. sotto* and related strains was investigated. The results of these tests are summarized in Table III, which shows that the larvae of several lepidopterous species are susceptible.

TABLE III

PATHOGENICITY OF CERTAIN BACTERIA* FOR LEPIDOPTEROUS LARVAE

Species of insect	Percentage mortality following massive doses					
	Bs.	Bt.	S1	S4	S2	Bc.
Bombyx mori (L.)	100	100	100	100	0	0
Anisota rubicunda (F.)	90	80	100†	100†	0†	18†
Anisota senatoria (A. and S.)	88	75	—	—	—	6
Nymphalis antiopa (L.)	85	75	—	—	—	7
Erannis tiliaria (Harr.)	100†		—	—	—	—
Datana integerrima (G. and R.)	100	73†	—	—	—	—
Datana ministra (Drury)	90		—	—	—	—
Liparis dispar L.	90		—	—	—	—
Protoparce quinquemaculata (Haw.)	100†		—	—	—	—
Protoparce sexta (Johan.)	100†		—	—	—	—
Cirphis unipuncta (Haw.)	20		—	—	—	—
Lambdina fiscellaria (Guen.)	0	0	—	—	—	—

*Bs.   *Bacillus sotto*   S1   *Bacillus sp.* (*Sporeine I*)
  Bt.   *Bacillus thuringiensis*   S4   *Bacillus sp.* (*Sporeine 4X*)
  Bc.   *Bacillus cereus ATCC No. 9818*   S2   *Bacillus sp.* (*Sporeine 2*)
†*10–30 insects tested; in others 50–200.*

Discussion

The results indicate that *B. sotto* and *B. thuringiensis* are closely related to *B. cereus* and cannot be differentiated by use of the usual biochemical tests. They differ in certain cultural characteristics, which are consistent and easily recognizable. The two also display morphological differences, the more important being that the vegetative cells of *B. sotto* are longer and narrower than those of *B. thuringiensis*. If it were not for the fact that crystals are absent from *B. cereus* there would seem to be considerable merit in Toumanoff's (26) proposal that *B. sotto* be reduced to a variety of *B. cereus*, as Smith, Gordon, and Clark (19) have suggested for *B. thuringiensis*, *B. anthracis*, and *B. mycoides*. However, since Smith has now withdrawn their proposal (personal communication) it seems preferable to retain the older name until the situation is clarified.

Sporulated cultures of *B. sotto* are more pathogenic for silkworm larvae than are cultures of *B. thuringiensis* grown under similar conditions. The symptoms in insects fed cultures of either are identical but it is not known if

the paralysis-inducing agent in each case is the same.   It is possible that *B. sotto* cultures may contain relatively more of the toxic agent or that the toxic agent produced by *B. sotto* may be more toxic, weight-for-weight, than the compound produced by *B. thuringiensis*.   The very small amount of culture material required to induce symptoms in silkworm larvae and the nature of the observed symptoms suggest the action of a neurotoxin.

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ASSOCIATION OF TOXICITY WITH PROTEIN-CRYSTALLINE INCLUSIONS OF BACILLUS SOTTO ISHIWATA[1]

By T. A. ANGUS[2]

Abstract

The toxic principle in sporulated cultures of *Bacillus sotto*, which causes paralysis and death in *Bombyx mori* larvae, is associated with the crystalline inclusions produced by this microorganism. The toxin is not present as a typical exotoxin but is soluble either in silkworm gut juice or dilute alkali solutions.

Introduction

The characteristics and the pathogenicity of *Bacillus sotto* Ishiwata, a bacterial pathogen of *Bombyx mori* L., the silkworm, have been discussed in a previous paper (2).

Old cultures of *B. sotto* and some other bacterial strains are more pathogenic for silkworm larvae than young cultures (3, 11, 12, 13). Aoki and Chigasaki (3) noted that agar cultures of *B. sotto* were only slightly toxic after incubation for two or three days, but that their toxic action was very marked after 9 or 10 days. They refer to these toxic cultures as "old" cultures and it is assumed that by this term they mean cultures that have sporulated, for in studies earlier reported (2) it was found that cultures of *B. sotto* on nutrient agar begin to sporulate after 36 hr. incubation at 28° C.

Aoki and Chigasaki (3) could not correlate the lethal action of *B. sotto* with the multiplication of bacteria ingested by silkworm larvae. They found that young vegetative cells were destroyed in the insect gut, and that although spores survived, they did not germinate. From this it was concluded that larvae that were fed old cultures of *B. sotto* were killed through the action of a substance already present in the culture. This toxic substance was not present in the medium in which the bacteria were grown, and was not filterable through clay candles, but boiling the culture for 10 min. destroyed its toxic activity as did iodine, formaldehyde, mercuric chloride, and alcohol.

In 1954, it was reported in a preliminary note (1) that the toxin theory of Aoki and Chigasaki was confirmed, and that the toxin, as suggested by Hannay (8), was associated with the crystalline inclusions produced by certain *Bacillus* species. The studies supporting these conclusions are reported here in detail. All the toxicity tests were made on silkworm larvae, using methods described previously (2).

Experimental

The work which is reported below in the sections dealing with the correlation between spore formation and toxicity, and with the localization and extraction

of the toxic principle of *B. sotto*, was completed before Hannay (8) drew attention to crystalline inclusions and their possible significance. The work in the final section of the paper was completed afterwards. The cultures used in the earlier work must have contained not only spores but crystals as well although the presence of the latter was not suspected nor was their significance in toxicity realized. These earlier experiments and results are described since they indicate that the spore and any product directly connected with spore germination cannot be responsible for the toxicity of *B. sotto* cultures, thus supporting the conclusion that the toxic principle in *B. sotto* cultures is associated with the crystalline inclusions.

Correlation between Spore Formation and Toxicity

The association of toxicity with "old cultures" (3) of *B. sotto* indicated the need for a study of the correlation between spore formation and toxicity. *B. sotto* was grown on a number of different media to determine the conditions under which sporulation would occur, and toxicity tests were made of the cultures. In general, it was found that sporulation would occur on any solid medium adequate for vegetative growth. It was also found that silkworm larvae became paralyzed after ingesting a quantity of a suspension of a sporulated culture. Cultures tested before sporulation occurred were not toxic.

Broth Cultures

Vegetative growth of *B. sotto* in still-broth cultures was abundant but after 100 hr. incubation at 28° C. only a few spores were to be seen in nigrosin films (Robinow's method, as described in (7)). That these cultures were weakly toxic could be shown only by centrifuging the culture and resuspending the sediment in a fraction of the original volume.

Since *B. sotto* is an aerobic sporeformer, it was thought that the low incidence of spores in still-broth cultures might be attributable to low oxygen tension. To test this a culturing apparatus was made, consisting of a 1-liter flask to which two tubes were attached. The first was a small side-arm tube projecting from the neck of the flask and attached to a suction line. When suction was applied air was drawn through a Seitz filter and bubbled into broth through a fritted glass tip. Foaming was controlled with octyl alcohol or "Dow Anti-foam C"*. The second tube, attached to the body of the flask, was used to remove samples. The use of the aeration flask permitted accurate sampling because the turbulence caused by the air stream kept the culture homogeneous, whereas in colonies growing on nutrient agar the proportion of sporulated and vegetative cells varies regionally.

Samples were tested for pathogenicity and examined by dark-field microscopy. It was found that cultures caused paralysis only after sporulation had begun. In one culture incubated at 30° C., pre-spores and a few mature spores were seen after 67 hr. and this culture caused paralysis. Another

Dow Chemical Company of Canada, Ltd. kindly supplied samples of this material.

culture incubated at a lower temperature (25° C.) contained only vegetative rods at 67 hr. and did not cause paralysis; pre-spores and mature spores were not seen until after 90 hr. incubation and the culture was then toxic.

Flasks containing nutrient broth were inoculated with *B. sotto* and shaken at 300 oscillations per minute on a rotary shaking machine at 28° C. At appropriate times the shaker was stopped and samples were withdrawn. These were tested for toxicity and the appearance of the cells was determined under a dark-field microscope. The results obtained were similar to those for aerated broth cultures. Only sporulated cultures were toxic.

The results of these culture studies indicate that toxicity is correlated with sporulation, for only cultures containing spores caused paralysis in larvae. This agrees with Hannay's observation that crystals occur in sporulated cultures (8).

Localization of Toxicity in *B. sotto* Cultures

Non-toxicity of Culture Filtrates

Various workers have reported that filtrates of broth cultures of some bacteria can be toxic for insect larvae (12, 15). An aerated culture of *B. sotto*, causing paralysis of silkworm larvae, was divided into two portions. Half of the culture was filtered through Seitz filter pads or fritted glass filters (Corning U.F.) and the filtrate tested for toxicity. It was not toxic. The remainder of the culture was centrifuged at 12,000 r.c.f. (10,000 r.p.m. in Sorvall SS-1A) for 30 min., and the toxicity of the sediment and the supernatant fluid tested. The sediment caused paralysis; the supernatant fluid did not. The centrifugation and filtration experiments showed that the paralysis-inducing agent is not present as a typical exotoxin. This is consistent with the finding reported below that the toxic principle and the crystals are soluble only in alkaline solutions.

Non-toxicity of Wash Waters

Toumanoff and Vago (14) suspended old cultures of *B. cereus* var. *alesti* in distilled water or physiological saline, centrifuged this suspension, and tested the sediment and the supernatant fluid for toxicity. Both fractions were toxic for silkworm larvae, and they concluded that the toxic substance is liberated in part in the distilled water or the physiological saline used as a suspending medium.*

Attempts were made to demonstrate a water soluble or physiological saline soluble toxin in *B. sotto* cultures. The method of centrifuging differed from that of Toumanoff and Vago (14), who centrifuged their suspensions once at 3000 r.p.m. for five minutes. In the present studies, the suspension was centrifuged at 12,000 r.c.f. (10,000 r.p.m. in Sorvall SS-1A) for 15 to 30 min. and the supernatant fluid was decanted and centrifuged again. When tested

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*"*La substance toxique est donc libérée en partie dans l'eau distillée ou dans l'eau physiologique ayant servi pour cette suspension.*"

for toxicity, the sediment caused paralysis; the supernatant fluid did not.   It was concluded therefore that the toxic principle of *B. sotto* is not soluble in water or physiological saline.

Toxicity of Suspensions of Smashed Spores

The preceding results did not exclude the possibility that toxic material was contained inside the spore, and that rupturing of the spore coat might make it possible to obtain it in solution or suspension.   The usual methods for rupturing bacterial cells are not effective with *Bacillus* spores, but Fitz-James (7) has reported the rupture of spores in a Mickle disintegrator, and an apparatus of this type was used in the experiments described below.   The methods developed by Fitz-James were modified as needed.

B. sotto was grown on nutrient agar at 28° C. for 150 hr., at which time sporulation was complete.   The culture was harvested, washed several times by centrifugation, resuspended in 5 ml. of cold sterile water, and mixed with 18 ml. of ballotini (0.1 to 0.2 mm.) in the Mickle chamber.   The machine was set for maximum amplitude and shaking carried on for two hours at 3° C. The suspension was filtered through a fritted glass filter (Corning C), and films of the suspension were prepared and examined by dark-field microscopy. The suspension was composed mostly of spore fragments and debris (part of which was undoubtedly made up of crystals whose presence and significance was not suspected when the experiments were made) and only an occasional intact spore could be seen.   Plating showed that the viable spore count had been reduced from 10^9 to 10^5 viable spores per ml. of suspension.

The suspension was centrifuged and the fractions were tested for toxicity; the sediment was toxic but the supernatant fluid was not.   A quantity of the toxic sediment was suspended in water and in physiological saline, and the mixtures were incubated, centrifuged, and tested for toxicity.   The sediments were still toxic but the supernatant fluids were not, indicating that although the spores were ruptured a toxic extract could not be obtained with water or physiological saline.

The pathogenicity of a diluted suspension of smashed spores (and intact crystals) was established quantitatively.   Each larva was fed foliage coated with 0.1 ml. of suspension containing about 2000 viable spores (usually a smaller number of spores was ingested since in most cases the test larvae ceased feeding before the entire coated leaf was consumed).   The suspension was toxic at this dosage.   The LD_{50} of *B. sotto* for *B. mori* larvae established earlier (2) contained about 50,000 viable spores per larva.   The fact that the suspension contained only 2000 viable spores per dose but was still toxic indicates that the toxicity is due to something other than intact viable spores.

In a further experiment, suspensions of intact and of smashed spores were tested for toxicity in ingestion and injection tests.   The number of viable spores in each suspension was established by plating.   A sterile microsyringe was used to inject 0.005 ml. of suspension into the body cavity of silkworm larvae; the same quantity was fed to larvae on coated leaves.   Heated

TABLE I

TOXICITY OF SUSPENSIONS OF SMASHED AND WHOLE CELLS OF *B. sotto*

Fraction	Dose in ml. per larva	No. of viable spores	No. of larvae	Paralysis (P) or death (D) in						
				2	3	4	6	12	22	30 hr.
Ingested										
Control* intact	0.005	—	5	0	0	0	0	0	0	0
Control smashed	0.005	—	5	0	0	0	0	0	0	0
Intact	0.005	850,000	10	1 P	5 P	9 P	9 P	9 P	9 D	9 D
Smashed	0.005	30	10	5 P	5 P	5 P	8 P	8 P	9 D	9 D
Injected										
Control intact	0.005	—	5	0	0	0	0	0	0	0
Control smashed	0.005	—	5	0	0	0	0	0	0	0
Intact	0.005	850,000	10	0	0	0	0	4 D	9 D	9 D
Smashed	0.005	30	10	0	0	0	0	0	2 D	8 D

Controls are heated suspensions.

suspensions (125° C. for 30 min.) of intact and of smashed spores were used as controls. The results (Table I) indicate that smashing does not alter the toxicity of *B. sotto* suspensions. In the ingestion tests paralysis occurred in from two to six hours; in the injection tests there was no paralysis but the viable spores remaining in the suspensions caused septicemia. These results indicate that the toxic action of *B. sotto* cultures is not dependent on the germination of spores in the gut of the host insect. This is consistent with the observation of Aoki and Chigasaki (3) that ingested spores of *B. sotto* do not germinate in the gut of silkworm larvae.

Extraction of Toxic Principle of *B. sotto*

*Extraction with Gut Juice**

Bolle (6) showed that the protein inclusion body of silkworm polyhedrosis was dissolved by gut juice, and this suggested that the toxic principle of *B. sotto* might be activated or released in a similar fashion. Tests were made to determine if this was so.

Fifth-instar larvae, starved for 12 hr., were bled by snipping off one of the abdominal prolegs. After bleeding ceased, a small incision was made in the body wall so that the gut extruded. The gut was pierced and the contents were collected in a tube immersed in an ice bath. Before use the gut contents were clarified by centrifugation (Sorvall SS-1A, 10,000 r.p.m. for 30 min.). The collected material was used immediately or stored at −30° C. *B. sotto* was grown on nutrient agar for 300 hr. at 28° C., harvested, and centrifuged. A quantity of the sedimented spores and debris (2 gm. wet weight) was suspended in gut juice (15 ml.) and incubated for six hours at 28° C. This suspension was centrifuged (10,000 r.p.m. for 30 min.) and the sediment and supernatant fluid were tested for toxicity. Both the sediment and supernatant fluid caused paralysis in silkworm larvae. The toxic supernatant fluid was

Gut juice is the fluid remaining after the solid material of the gut contents has been removed by centrifugation.

TABLE II

TOXICITY OF GUT JUICE EXTRACTS OF A SPORULATED CULTURE OF B. *sotto*

Fraction	No. of larvae tested	Amount in ml.	Death (D) or paralysis (P) in		No. of viable spores per dose
			4 hr.	24 hr.	
Gut juice	10	0.1	0	0	—
Sediment	10	0.1	10 P	10 D	t.n.t.c.*
Supernatant fluid	20	0.1	20 P	20 D	250
Filtered supernatant fluid	10	0.1	10 P	10 D	Sterile
	65	0.1	46 P	46 D	Sterile
Heated supernatant fluid	10	0.1	0	0	Sterile

t.n.t.c. = too numerous to count.

then filtered through a fritted glass filter (Corning U.F.). A portion of the unfiltered toxic supernatant fluid was heated at 100° C. for 30 min. and used as a control. The results (Table II) indicate that a cell-free toxic principle had been extracted from cultures of B. *sotto*.

Extraction with Dilute Alkali

In some regions of the gut, the gut contents of *Bombyx mori* larvae are markedly alkaline (pH 9.5–10.0), and this suggested an extraction based on alkali. There were grounds for believing that this would be successful, for Bergold (4) had found that viral polyhedral protein which is soluble in silkworm gut juice is also soluble in alkali. In the present studies a carbonate buffer, pH 10.3, 0.2 M was used. A sporulated culture of B. *sotto* was harvested, washed, and resuspended (500 mgm. wet weight) in 20 ml. of buffer solution. This mixture was incubated for three hours at 28° C., centrifuged, and the fractions were tested for toxicity. The sediment was still toxic for larvae as before, but the supernatant fluid also was toxic. The dilute alkali solution had dissolved some part of the suspension making it possible to obtain a solution of the toxic principle.

Association of Toxicity with Crystalline Inclusions

Berliner (5) in 1915, and later Mattes (10) in 1927, described a structure in sporulated B. *thuringiensis* cultures that Mattes called "Restkorper", but made no suggestions regarding its function. In 1954, during a study of spore formation in B. *thuringiensis*, Hannay (8) noted that the spores were invariably accompanied by what appeared to be diamond-shaped crystals and suggested that they might be implicated in insect disease. These crystals were present in some other bacterial pathogens of insects, but not in B. *cereus* strains known to be non-pathogenic for insects. A most important observation, from the point of view of these studies, was that the crystals were insoluble in water or physiological saline but readily soluble in dilute alkali.

Hannay's observations (8) indicated that two questions should be answered with respect to *B. sotto*; do sporulated cultures contain crystalline inclusions and, if so, what is the effect on these bodies of the dilute alkali used in preparing sterile toxic solutions?

Effect of Dilute Alkali on B. sotto Crystalline Inclusions

Cultures of *B. sotto* grown on nutrient agar for various times were examined as described by Hannay (8), and it was seen that *B. sotto* produces crystalline inclusions of the same shape as those of *B. thuringiensis* (9). Moreover, further studies showed that crystalline inclusions were always associated with sporulation in *B. sotto*.

The effect of dilute alkali on the spores and crystalline inclusions in *B. sotto* cultures was studied. Sporulated cultures were washed three times by centrifuging, and resuspended in water. A sample of the suspension of washed crystals and spores was stored at 3° C. and used for microscopic examination and for plate counts. To the remainder, sufficient 1 N sodium hydroxide solution was added to give a final normality of 0.1 N, and the mixture was centrifuged and the supernatant fluid set aside. The sediment was washed twice in cold sterile water by centrifuging and resuspended in cold sterile water. Samples were tested for numbers of viable spores, and for toxicity, and examined in nigrosin films. The washed alkali-treated spores were still viable, and on culturing gave rise to colonies that eventually sporulated and produced crystalline inclusions. The sediment was only very weakly toxic. Examination of nigrosin films of the sediment showed that the crystals had become less refractile. Hannay and Fitz-James (9) found that the crystalline inclusions of *B. thuringiensis* became less refractile after treatment at pH 10.7.

The alkaline supernatant fluid was centrifuged again and sterilized by filtration through a fritted glass filter (Corning U.F.). Before toxicity tests the filtrate was dialyzed against running water for 72 hr. to remove the alkali. A portion of the filtered dialyzed supernatant fluid was heated at 70° C. for 30 min. and used as a control. All the fractions were tested by feeding and injection. The results are given in Table III, which is adapted from one

TABLE III

The effect of feeding and injecting larvae of *Bombyx mori* with fractions of an alkali-treated culture of *B. sotto*[*]

Fraction tested	Viable spores per larva	Method of testing	
		By feeding	By injection
Original suspension	10⁶	Paralysis within 4 hr.	Septicemia within 12 hr. but no paralysis
Sediment of washed alkali treated spores	10⁷	No effect	Septicemia within 12 hr. but no paralysis
Filtered supernatant fluid	Sterile	Paralysis within 4 hr.	No effect
Dialyzed filtrate	Sterile	Paralysis within 4 hr.	No effect
Heated dialyzed filtrate	Sterile	No effect	No effect

Adapted from Angus (1).

previously published (1). From the results it is concluded that treatment of a mixture of *B. sotto* spores and crystals with dilute alkali alters the appearance of the crystals and extracts a paralysis-inducing principle. This could be regarded as evidence that the toxic principle was associated with the crystals, but the possibility remained that the toxic principle was adsorbed on the spore or associated with the cellular debris rather than the crystals.

Preparation of Pure Crystal Preparations

Hannay and Fitz-James (9) described a method for separating the spores and crystals of *B. thuringiensis* that took advantage of the tendency of spores to germinate spontaneously and autolyze. When this occurred the crystals were separated from the spore debris by differential centrifugation. This phenomenon also occurs in cultures of *B. sotto*, and a similar method was used to prepare a pure crystal suspension. Nigrosin films were prepared from the suspension, and the number of crystals and spores counted in several microscope fields; about 99% were crystals.

The suspension of crystals was washed another 10 times in distilled water, three times in 1 M saline, incubated for one hour at 37° C. in 1 M saline, and then finally washed another 10 times in distilled water. The purpose of these repeated washings was to remove any material adsorbed on the surface of the crystal. The cleaned crystals in suspension were sedimented by centrifugation, the pellet was frozen, dried over silica gel for 72 hr., and then over phosphorus pentoxide for a further 72 hr. The dried crystals were transferred to weighing bottles, weighed, resuspended in a measured volume of double distilled water, and stored at 3° C.

Toxicity of Crystalline Inclusions

A quantity of the crystal suspension was diluted serially and tested for toxicity against silkworm larvae. An equal amount of each dilution was plated, and the number of viable spores per dose determined. The results of a typical test are given in Table IV. As may be seen from the results, the crystalline inclusions were toxic. In a previous experiment (Table III) 10^7 viable spores (freed of crystals by alkali treatment) had no effect on larvae.

TABLE IV

TOXICITY OF *B. sotto* CRYSTALLINE INCLUSIONS FOR SILKWORM LARVAE

Weight in μgm. crystals per gram larva	Approx. no. of viable spores per larva	No. of larvae	Paralysis (P) or death (D) in		
			5 hr.	9 hr.	24 hr.
2.0	150	14	14 P	14 P	14 D
1.0	75	12	12 P	12 P	12 D
0.75	50	7	7 P	7 P	7 D
0.45	40	13	11 P	12 P	12 D
0.25	20	16	10 P	10 P	10 D
0.16	15	12	5 P	7 P	7 D

It seems reasonable to assume that the small number of viable spores (15–150) present in the doses of suspended crystals could not be responsible for the observed mortality. It is concluded, therefore, that the toxic component in sporulated cultures of *B. sotto* is associated with the crystalline inclusions, and that Hannay's theory that the crystals are "in some way connected with the formation of a toxic substance encouraging septicæmia of the insect larvae" (8) is confirmed.

Conclusions based on the results given in Table IV must be qualified, especially as they relate to the smaller doses.   When very clean crystals are freeze-dried and resuspended, the suspension contains many clumps of crystals. Subject to the error introduced by clumping, the results indicate that the LD_{50} is less than 0.5 μgm. dried crystals per gram larva.

The composition of the crystalline inclusions of *B. sotto* and of a product derived from them will be given in a subsequent paper.   Preliminary chemical analyses indicate that the crystals are largely protein in nature.

Conclusions

The paralysis-inducing principle of *B. sotto* is present only in sporulating cultures; it is not a typical exotoxin, nor can it be extracted by treatment with water or physiological saline from whole or smashed cultures.   The typical paralysis is not caused by the growth of the microorganism in the host tissue. The toxic principle is soluble in insect gut juice or dilute alkali and is associated with the crystalline inclusions.   In the present study no extensive investigation has been made of the factors involved in the production of crystalline inclusions in *B. sotto* beyond establishing that it is always a concomitant of sporulation.

The results constitute a confirmation of the work of Aoki and Chigasaki, and of Hannay's suggestion that the crystalline inclusions found in some bacterial pathogens of insects might be involved in the diseases caused by these organisms.

Acknowledgments

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ANAEROBIC CONVERSION OF D-XYLOSE TO TRIOSE PHOSPHATE AND HEXOSE PHOSPHATE BY EXTRACTS OF PSEUDOMONAS HYDROPHILA[1]

By R. M. HOCHSTER AND B. A. STONE[2]

Abstract

By analysis of reaction mixtures containing D-xylose, adenosinetriphosphate, and cell-free sonic extracts of *Pseudomonas hydrophila*, evidence has been obtained for the formation of triose and hexose phosphates under anaerobic conditions. The hexose phosphate fraction was shown to be a mixture of fructose-6-phosphate and glucose-6-phosphate by chromatographic and by enzymatic methods. The time course of the formation and disappearance of pentose, heptulose, triose, and hexose phosphate intermediates was also determined. These data, together with the results of previous work from this laboratory, have been combined to present an over-all scheme of enzymatic reactions for the anaerobic conversion of D-xylose to triose and hexose phosphates.

Introduction

In previous publications from this laboratory (9, 10) evidence was presented to show that cell-free sonic extracts of *Pseudomonas hydrophila* convert D-xylose to sedoheptulose phosphate under anaerobic conditions utilizing adenosinetriphosphate (ATP) as phosphate donor. These experiments were carried out with thoroughly dialyzed preparations under conditions selected to be most favorable for the accumulation of early intermediates in order to facilitate their identification. The intermediates found were: D-xylulose, D-xylulose-5-phosphate, D-ribulose-5-phosphate, and D-ribose-5-phosphate. The same equilibrium mixture of D-xylulose-5-phosphate and D-ribulose-5-phosphate resulted whether D-xylose plus ATP or D-ribose-5-phosphate was used as initial substrate. The xylulose–ribulose interconversion was attributed to a process of enzymatic epimerization, the functioning of which has also been suggested more recently by Dickens and Williamson (5) and by Horecker (12).

Our studies had also shown that in less thoroughly dialyzed preparations the amount of pentose phosphate accumulating was much smaller (10). The results of experiments with undialyzed preparations in which D-xylose was converted anaerobically to triose and hexose phosphates are reported in the present paper.

Materials and Methods

D-Xylose and D-ribose-5-phosphate (Ba salt) were purchased from Nutritional Biochemicals Corp., D-glucose-6-phosphate (Ba salt), D-fructose-6-phosphate (Ba salt), D-fructose-1,6-diphosphate (Ba salt), and monosodium glutathione from Schwarz Laboratories Inc. ATP was the product of Pabst Brewing Co.

and anthrone was obtained from Matheson Co. Inc.   Sedoheptulosan mono-
hydrate was kindly donated by Dr. N. K. Richtmyer, National Institutes of
Health, Bethesda, Md., and DL-glyceraldehyde-1-bromide-3-phosphoric acid
dioxane addition compound by Dr. E. Baer, University of Toronto.

Enzyme Preparations
Sonic Preparation

Cells of *Pseudomonas hydrophila* (NRC 492) grown in 24-hr. shake cultures
on a synthetic medium (11) were harvested and washed three times with
0.9% sodium chloride.   Twelve grams of cells (wet weight) were suspended
in 30 ml. of a solution of reduced glutathione (0.75 gm. in 150 ml., pH 7.5) and
treated in a sonic oscillator (Raytheon 10 kc.) at 1.1 amp. for 20 min.   The
resulting preparation was centrifuged at 7500 r.p.m. (5900 $\times$ g) in a Spinco
preparative centrifuge for 30 min. at 0–4° C. to remove unbroken cells and cell
debris.   The turbid, slightly reddish opalescent supernatant was used for the
experiments and will be referred to as the "sonic preparation".

Charcoal-treated Preparation

The sonic preparation was freed of pyridine nucleotides (24) by gentle
stirring at 0–4° C. for one hour with acid-washed, air-dried charcoal (Darco
G-60).   The ratio of charcoal to extract was 100 mgm. for every 7.5 ml.   The
charcoal was then removed by centrifugation at 5000 r.p.m. (2000 $\times$ g) for
one hour at 0–4° C. in a Spinco preparative centrifuge.   The supernatant was
used for the experiments.

Other Enzymes

Acid phosphatase was prepared as described previously (10), glucose-6-
phosphate dehydrogenase was supplied by Mann Research Laboratories,
and crystalline glyceraldehyde phosphate dehydrogenase by Nutritional
Biochemicals Corp.

Analytical Methods

Barium–zinc precipitation (20) was used to remove sugar phosphates and
nucleotides from solution (8) prior to the estimation of residual, free pentose.
ATP and adenosinediphosphate (ADP) were specifically removed by precipita-
tion with mercuric acetate (8) and the supernatant solutions used to estimate
heptulose phosphate and total pentose.   Triose phosphate and hexose
phosphate were determined in trichloroacetic acid filtrates.

Free pentose was estimated by the method of Mejbaum (18) using 40 min.
heating periods (1).   Total pentose (free pentose plus pentose phosphate)
and heptulose were determined by the method of Horecker, Smyrniotis, and
Klenow (13) and their concentrations calculated by solution of the simul-
taneous equations given by these authors.   From the total pentose values
obtained by this method, the pentose phosphate was calculated by subtraction
of the free pentose values.   Triose phosphate was determined as alkali-labile

phosphate using the method of King (15).   Assays for hexose phosphate were carried out by the anthrone method using the same heating time and anthrone solutions as given by Trevelyan and Harrison (21).   Readings were taken at 620 mμ.   The concentration of sulphuric acid used (71.5 vol. %) is well below the value (96.4 vol. %) which Johanson (14) has found to cause a shift in absorption maximum and below the value of 81 vol. % which was found to give no shift.   Some interference by pentose and heptulose was, however, found to exist in the anthrone assay for hexose.   Suitable correction was made following experiments on color development with this reagent in the presence of known mixtures of hexose and pentose and of hexose and heptulose. Glucose-6-phosphate was assayed with glucose-6-phosphate dehydrogenase as described by Kornberg and Horecker (16).   Protein nitrogen was determined (N $\times$ 6.25) by the Kjeldahl method of McKenzie and Wallace (17).

Paper Chromatographic Procedure

Descending chromatography was carried out on Whatman No. 1 filter paper and sugars were detected as previously described (10).   Solvents used were: (A) propanol – acetic acid (glacial) – water (60 : 1 : 39) and (B) acetone– pyridine–water–ammonia (80 : 20 : 4 : 1).

Experimental and Results

Reaction mixtures were incubated in conventional Warburg flasks in an atmosphere of 95% nitrogen and 5% carbon dioxide at 27° C.   The flasks contained: 0.015 M tris(hydroxymethyl)aminomethane (Tris) buffer pH 7.4, 0.0166 M ATP, 0.8 ml. sonic preparation (or 0.91 ml. charcoal-treated preparation), and 0.0033 M D-xylose in a total volume of 3.0 ml.   The substrate was added from the side arm at zero time.   At desired time intervals, the contents of duplicate vessels were combined and the reactions stopped by addition of one of several precipitant solutions to aliquots of the reaction mixtures.   The supernatants were then used for the analysis of the components as indicated in *Analytical Methods*.

Charcoal-treated as well as untreated enzyme preparations were used in these experiments.   Comparison of the results from the two systems was thus possible in this and subsequent papers to be published.   The amount of each enzyme preparation was adjusted to give equal final protein nitrogen content.

Table I shows the disappearance of free xylose and Fig. 1A summarizes the amount of the various sugar phosphate components appearing and disappearing in the course of the incubation.

Several conclusions may be drawn from these results.   The initial formation of pentose phosphates (at 10 min.) coincides with the initial drop in free xylose content.   At this point the formation of other components is still very small. Soon after, pentose phosphates decline with the concomitant formation of heptulose, triose, and hexose phosphates.   Heptulose phosphate reaches a maximum (60 min.) and thereafter disappears at approximately the same rate

as the pentose phosphate.   Finally, hexose and triose phosphates predominate. The system with charcoal-treated enzyme showed a very similar pattern (Table I and Fig. 1B).

Alkali-labile phosphorus determinations were done following incubation of the aliquots with 1.0 N KOH for 20 min. at room temperature.   That the triose in question was, in fact, D-glyceraldehyde-3-phosphate was demonstrated by carrying out standard spectrophotometric analyses using crystalline glyceraldehyde phosphate dehydrogenase, DPN, and arsenate (23).

In order to obtain information on the nature of the hexose phosphate, xylose was incubated with a fresh sonic enzyme preparation in the manner described above.   At zero time and at 60 min., the contents of three Warburg vessels were combined, heated at 100° C. for three minutes, chilled, the denatured protein centrifuged down, and the supernatant concentrated to dryness at 30° C. in a Craig evaporator (3).   To each sample 0.5 ml. water

TABLE I

DISAPPEARANCE OF D-XYLOSE DURING ANAEROBIC INCUBATION
IN THE PRESENCE OF ATP

	D-Xylose remaining (μM.)	
Time (min.)	Sonic preparation	Charcoal-treated preparation
0	20.0	20.0
10	14.9	14.2
60	12.5	12.1
120	10.4	10.1
240	9.0	8.9
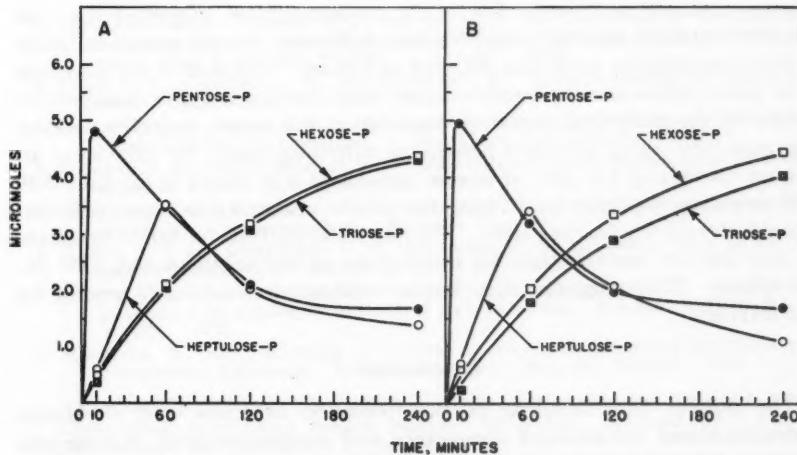
FIG. 1.   Time course of formation of sugar phosphates from 20 μM. D-xylose.   A, sonic preparation; B, charcoal-treated preparation.

was then added.   The solutions were streaked onto several filter papers and developed for 26 hr. using solvent system A.   After drying overnight, the hexose phosphate areas were determined by spraying sample strips with silver nitrate (22), aniline phthalate (19), and molybdate (7) and the appropriate areas cut out and eluted from the papers with distilled water.   Dephosphorylation was then carried out with acid phosphatase (10) at pH 5.3.   After incubation for one hour at 30° C., 2 ml. 20% trichloroacetic acid was added to each sample, the mixture centrifuged clear, and the two supernatants passed through mixed bed ion exchange resins (IR 120 (H$^+$) and IRA 400 (OH$^-$)). The effluents were concentrated to a sirup and 0.5 ml. water was added.   This material was then chromatographed with solvent system B overnight.

TABLE II

CHROMATOGRAPHIC IDENTIFICATION OF COMPONENTS OF
THE DEPHOSPHORYLATED HEXOSE
PHOSPHATE FRACTION

Materials	R_{gl}*
Glucose (control)	1.00
Fructose (control)	1.43
Acid phosphatase (control)	—
Zero time sample	—
60 min. sample	1.03, 1.42

$$*R_{gl} = \frac{Distance\ travelled\ by\ material}{Distance\ travelled\ by\ glucose}.$$

   The results of this experiment are shown in Table II.   They show that the original hexose phosphate area was composed of the phosphates of both fructose and glucose.   Inspection of the chromatograms suggested that the mixture contained relatively more fructose, however.   A more precise estimate of their quantitative ratio was obtained as follows.   Aliquots of the solutions were taken before the dephosphorylation step mentioned above, assayed for hexose by the analytical procedure described in this paper, and then assayed enzymatically using glucose-6-phosphate dehydrogenase.   In this way, an aliquot containing 1.0 μM. of hexose phosphate was shown to contain 0.38 μM. as glucose-6-phosphate.   Thus, after 60 min. reaction time the equilibrium strongly favors the fructose ester.   Furthermore, analysis for total phosphate in this fraction showed that for every mole of hexose there was 1.04 M. phosphate.   This suggests that hexose diphosphate was not present in the mixture.

Discussion

   The present demonstration of the formation of triose and of hexose phosphates and the order of appearance and disappearance of pentose and heptulose phosphates in extracts of *P. hydrophila* using D-xylose as the substrate extends the anaerobic pathway established earlier (10) for the conversion

of this pentose to ribose-5-phosphate and sedoheptulose-7-phosphate.   The results are best explained by assuming that xylose, following its conversion to xylulose-phosphate, and epimerization of the latter to ribulose-phosphate, enters the series of transketolase–transaldolase (6, 12) reactions which give rise to the net formation of triose and hexose phosphates consistent with the scheme recently proposed (4).   These reactions are summarized below.*
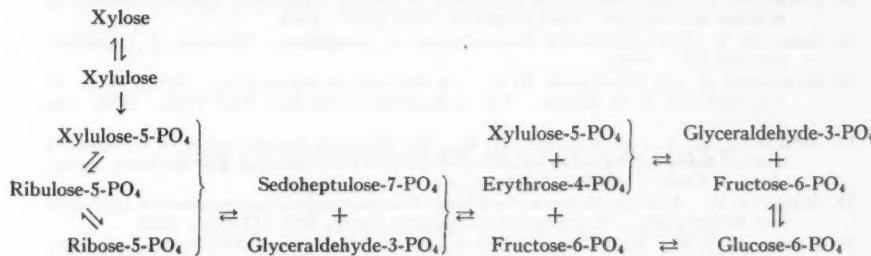
Essentially similar conclusions have recently been reached by Altermatt *et al.* (2) studying the end products of the anaerobic dissimilation of xylose by whole cells of *Aerobacter aerogenes* by means of the isotope tracer technique.

Acknowledgment

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*Note added in proof: *Short notes have just been published by P.A. Srere, J.R. Cooper, V. Klybas, and E. Racker (Arch. Biochem. Biophys. 59:535–538. 1956) and by B.L. Horecker, J. Hurwitz, and P. Z. Smyrniotis (J. Am. Chem. Soc. 78: 692–694. 1956) which clearly indicate that xylulose-5-PO_4, but not ribulose-5-PO_4, is one of the donor substrates in the reactions catalyzed by transketolase. ·This new observation has been taken into account in the formulation of the above scheme.*

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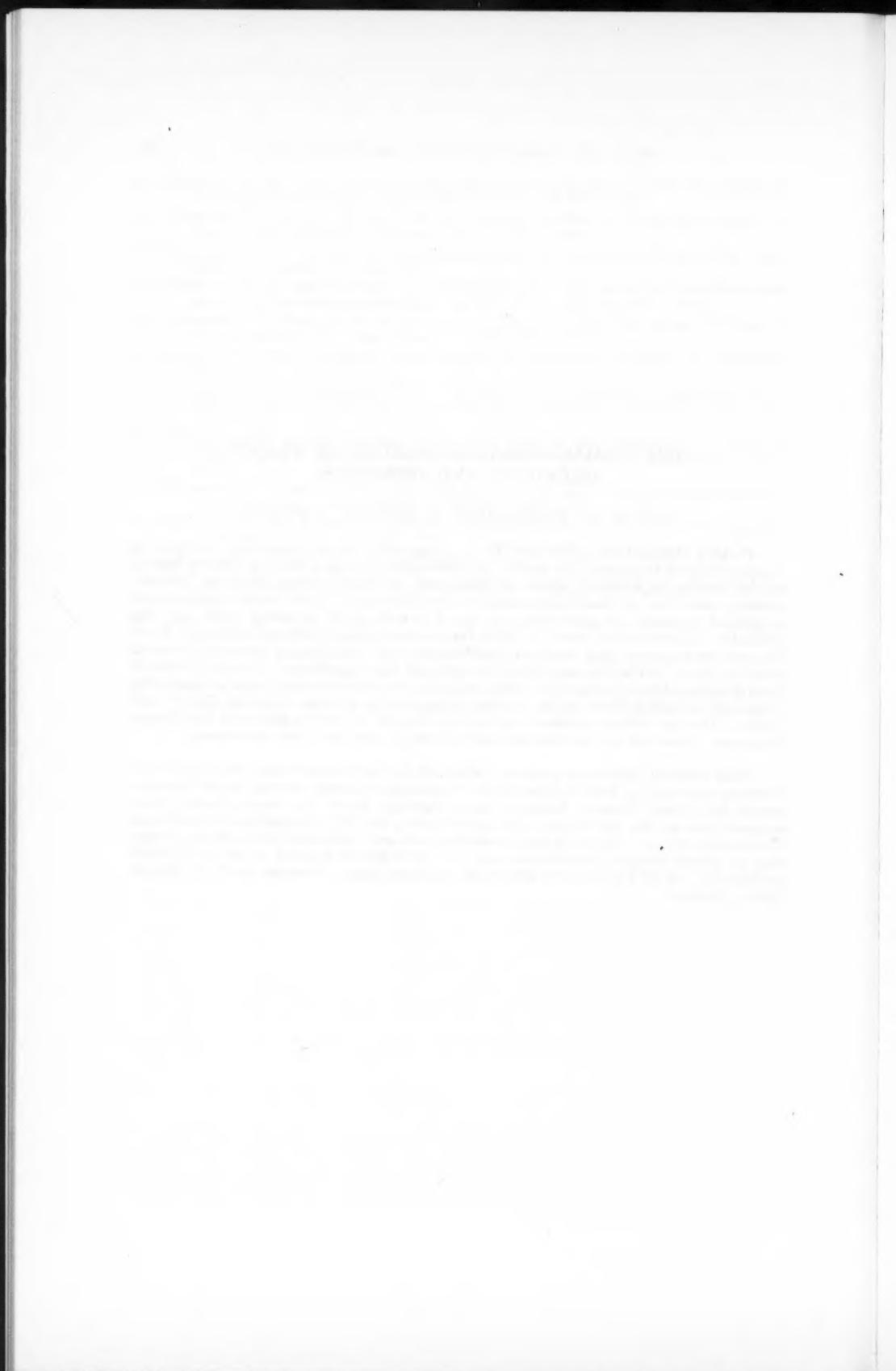
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    Manuscripts, in English or French, should be typewritten, double spaced, on paper $8\frac{1}{2} \times 11$ in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

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    An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

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